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(54) Title: METHODS OF INHIBITING ECTOPIC CALCIFICATION

(57) Abstract

The invention provides a method of inhibiting ectopic calcification in an individual. The method consists of administratering to the invidual a therapeutically effective amount of osteopontin or a functional fragment thereof. The invention also provides a method of treating or inhibiting ectopic calcification by administering to an individual acid–producing cells targeted to the site of ectopic calcification. The invention further provides a method of treating or inhibiting ectopic calcification in an individual consisting of promoting recruitement of acid producing cells to a site of ectopic calcification by administering osteopontin. The invention also provides a method of treating or inhibiting ectopic calcification in an individual consisting of increasing expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin.

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METHODS OF INHIBITING ECTOPIC CALCIFICATION

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BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the field of medicine and, more specifically, to methods of inhibiting ectopic calcification.

BACKGROUND INFORMATION

Deposition of calcium crystals in tissues other than teeth or bone, referred to as ectopic calcification, commonly occurs in association with renal failure, cardiovascular disease, diabetes and the aging process. A frequent finding in patients with renal failure, particularly those undergoing long-term hemodialysis and unable to appropriately regulate serum mineral balance, is calcification of internal organs, including the lung, heart, stomach and kidneys. Less commonly, hemodialysis patients develop painful calcified skin lesions that progress to non-healing ulcers or gangrene and may require amputation of the affected limb.

Ectopic calcification is also a common complication of the implantation of bioprosthetic heart valves and is the leading cause of replacement valve

failure. Ectopic calcification also occurs in native heart valves and blood vessels in association with atherosclerosis, diabetes and cardiovascular disease. The deposition of minerals in the vasculature narrows the orifices and hardens the walls of the affected valves and blood vessels, resulting in reduced blood flow to the heart and peripheral organs. Therefore, ectopic calcification increases the risk of valve failure, stroke, ischemia and myocardial infarction.

One protein that is abundant at the sites of ectopic calcification, such as in atherosclerotic plaques and in calcified aortic valves, is osteopontin. Osteopontin has several known functions, including promoting cell adhesion, spreading and migration. Osteopontin colocalizes with sites of early calcification in coronary atherosclerotic plaques and its expression increases as atherosclerosis develops. These findings, combined with studies showing that osteopontin has calcium-binding properties *in vitro*, have led to the suggestion that osteopontin may be involved in ectopic calcification. Previous studies have not addressed the role of osteopontin in ectopic calcification *in vivo*.

Ectopic calcification, if left untreated, results in increased morbidity and death. Current therapies to normalize serum mineral levels or to inhibit calcification of vascular tissues or implants are of limited efficacy and cause unacceptable side effects.

Thus, there exists a need for an effective method of inhibiting ectopic calcification. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a method of inhibiting ectopic calcification in an individual. The method consists of administering to the individual a therapeutically effective amount of osteopontin or a functional fragment thereof. The method can be used to inhibit ectopic calcification associated with a variety of conditions such as atherosclerosis, stenosis, restenosis, prosthetic valve replacement, angioplasty, renal failure, tissue injury, diabetes and aging. The invention also provides a method of treating or inhibiting ectopic calcification by administering to an individual acid-producing cells targeted to the site of ectopic calcification. The invention further provides a method of treating or inhibiting ectopic calcification in an individual consisting of promoting recruitement of acid producing cells to a site of ectopic calcification by administering osteopontin. The invention also provides a method of treating or inhibiting ectopic calcification in an individual consisting of increasing expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of human osteopontin, as described by Kiefer et al., Nucleic Acids Res. 17:3306 (1989).

Figure 2 shows the effects of osteopontin (a) on calcification of BASMC as compared to vitronectin and fibronectin (b).

Figure 3 shows the effects of osteopontin on alkaline phosphatase activity of BASMC (a) and phosphorous concentration in the medium (b) and the effects of levamisole and osteopontin (OPN) on alkaline phosphatase (ALP) activity (c).

Figure 4 shows the effects of osteopontin on calcium deposition (a), medium phosphorous concentration (b) and medium calcium concentration (c) at various initial calcium concentrations.

Figure 5 shows the effects of recombinant osteopontin and its functional fragments on HSMC calcium deposition (a) and the extent of phosphorylation of recombinant osteopontin fragments by casein kinase II (b).

Figure 6 shows the effect of phosphorylation and dephosphorylation of osteopontin on HSMC calcification.

Figure 7 shows the effect of various concentrations of osteopontin on HSMC calcification.

Figure 8 shows the time course of osteopontin inhibition of HSMC calcification.

Figure 9 shows the effect of osteopontin gene copy number on calcification of valves implanted subcutaneously into mice.

Figure 10 shows the effect of osteopontin gene copy number on osteopontin accumulation on valves implanted subcutaneously into mice and explanted at 7, 14, and 30 day time points (a), and calcium deposition on

valves implanted subcutaneously into mice and explanted at 7, 14, 30, and 60 day time points (b).

Figure 11 shows the effect of osteopontin gene copy number on macrophage recruitment to the site of valves implanted subcutaneously into mice.

Figure 12 shows the effect of osteopontin gene copy number on pH of valves implanted subcutaneously into mice.

Figure 13 shows the effect of osteopontin gene copy number on carbonic anhydrase II expression at the site of valves implanted subcutaneously into mice.

Figure 14 shows the effect of osteopontin gene copy number on the number of multinucleate foreign body giant cells on valves implanted subcutaneously into mice.

Figure 15 shows the effect of osteopontin phosphorylation on valve mineralization *in vivo*.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to an effective method for the inhibition of ectopic calcification. Ectopic calcification commonly occurs in association with renal failure, cardiovascular disease, diabetes and the aging process. Ectopic calcification of the vasculature increases an individual's risk of myocardial infarction, ischemia, stroke, dissection after angioplasty and heart valve failure. Ectopic calcification of prosthetic implants, such as bioprosthetic heart valves, is the leading cause of implant failure. Therefore, the method

will reduce disease and death associated with ectopic calcification.

The method is based on the discovery that osteopontin is able to effectively and specifically inhibit ectopic calcification. Therefore, ectopic calcification can be prevented or treated by administering a therapeutically effective amount of osteopontin or a functional fragment thereof to an individual, either systemically or at the predicted or known sites of ectopic calcification. As osteopontin is normally found in calcified tissues and at the sites of ectopic calcification, it can be administered with minimal toxic or immunogenic side effects.

As used herein, the term "ectopic calcification" is intended to mean the abnormal deposition of calcium crystals at sites other than bones and teeth. Ectopic calcification results in the accumulation of macroscopic amorphous calcium phosphate and hydroxyapatite deposits in the extracellular matrix.

Ectopic calcification can occur in a variety of tissues and organs and is associated with a number of clinical conditions. For example, ectopic calcification can be a consequence of inflammation or damage to the affected tissues or can result from a systemic mineral imbalance. Commonly, ectopic calcification occurs in vascular tissue, including arteries, veins, capillaries, valves and sinuses. Inflammation or damage to the blood vessels can occur, for example, as a result of environmental factors such as smoking and high-fat diet. Inflammation or damage can also occur as a result of trauma to the vessels that results from injury, vascular surgery, heart surgery or angioplasty. Vascular

calcification is also associated with aging and with disease, including hypertension, atherosclerosis, diabetes, renal failure and subsequent dialysis, stenosis and restenosis.

Ectopic calcification also occurs in non-vascular tissues, such as tendons (Riley et al., Ann. Rheum. Dis. 55:109-115 (1996)), skin (Evans et al., Pediatric Dermatology 12:307-310 (1997)), sclera (Daicker et al., Opthalmologica 210:223-228 (1996) and myometrium (McCluggage et al., Int. J. Gynecol. Pathol. 15:82-84 (1996)), each of which is incorporated herein by reference. In diseases resulting in systemic mineral imbalance, such as renal failure and diabetes, ectopic calcification in visceral organs, including the lung, heart, kidney and stomach, is common (Hsu, Amer. J. Kidney Disease 4:641-649 (1997), incorporated herein by reference). Furthermore, ectopic calcification is a frequent complication of the implantation of biomaterials, prostheses and medical devices, including, for example, bioprosthetic heart valves (Vyavahare et al., Cardiovascular Pathology 6:219-229 (1997), incorporated herein by reference). The methods of the invention are applicable to ectopic calcification that occurs in association with all of these conditions.

The term "ectopic calcification" is not intended to refer to the calcification that normally occurs within the bone matrix during bone formation and growth. Ectopic calcification, as used herein, is also distinct from abnormal calcification that occurs in renal tubules and urine that results in the formation of primarily calcium oxalate-containing kidney stones.

As used herein, the term "inhibiting," in connection with inhibiting ectopic calcification, is intended to mean preventing, retarding, or reversing formation, growth or deposition of extracellular matrix hydroxyapatite crystal deposits.

As used herein, the term "osteopontin" is intended to mean a molecule that is able to inhibit ectopic calcification and that is recognizably similar to one or more molecules known in the art as osteopontin. Osteopontin is characterized as a phosphorylated sialoprotein having a predicted molecular weight of about 34 kDa. Due to high negativity, post-translational modifications and alternatively spliced isoforms, osteopontin has been reported to have an apparent molecular weight of between about 44 and 85 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Giachelli et al., Trends Cardiovasc. Med 5:88-95 (1995)). All of the post-translationally modified forms and alternatively spliced isoforms of osteopontin are included within the definition of osteopontin as used herein.

Osteopontin has been identified in various species, including rat (Oldbert et al., Proc. Natl., Acad. Sci. USA 83:8819-8823 (1986)); mouse (Craig et al., J. Biol. Chem. 264:9682-9689(1989)); human (Kiefer et al., Nucleic Acids Res. 17:3306 (1989) and Young et al. Genomics 7:491-502 (1990)); pig (Wrana et al., Nucleic Acids Res. 17:10119 (1989)); cow (Kerr et al., Gene 108:237-243 (1991)); rabbit (Tezuka et al., Biochem. Biophys. Res. Commun. 186:911-917 (1992)); and chicken (Moore et al., Biochemistry 30:2502-2508 (1991)), each of which is incorporated herein by reference. Osteopontin from these species and osteopontin homologs from other

vertebrates are included within the definition of osteopontin as used herein.

Osteopontin can be characterized by the presence of one or more domains that are conserved across known species. The conserved domains that characterize osteopontin include, for example, an N-terminal signal sequence, casein kinase II phosphorylation sites, an alternatively spliced domain, an Arg-Gly-Asp (RGD)-containing integrin-binding cell adhesion domain, an Asp-rich calcium binding domain, a calcium binding homology domain and two heparin binding homology domains (Giachelli et al., supra (1995)). Therefore, newly identified molecules that possess one or more of these characteristic features of osteopontin are also included within the definition of osteopontin.

Osteopontin is also known in the art as bone sialoprotein I, uropontin, secreted phosphoprotein I, 2ar, 2B7 and Eta 1 (Giachelli et al., supra (1995)). The molecules encompassed by all of these terms used in the art are included within the definition of osteopontin as used herein.

The nucleotide and deduced amino acid sequence for human osteopontin have been described by Kiefer et al., supra (1989), and are set forth herein as Figure 1 (SEQ ID NOS:1 and 2). The term osteopontin is intended to include, for example, polypeptides having substantially the same amino acid sequence as shown as SEQ ID NO:2 and encoded by substantially the same nucleotide sequence as shown as SEQ ID NO:1.

Modifications of osteopontin and its functional fragments that either enhance or do not greatly affect

the ability to inhibit ectopic calcification are also included within the term "osteopontin." Such modifications include, for example, additions, deletions or replacements of one or more amino acids from the native amino acid sequence of osteopontin with a structurally or chemically similar amino acid or amino acid analog. For example, the substitution of one or more phosphorylated amino acids, such as serine or threonine residues, by negatively charged amino acids, such as glutamic acid or aspartic acid, is contemplated. The substitution or addition of residues, such as kinase phosphorylation consensus sequences, that can be phosphorylated either *in vivo* or *in vitro* is also contemplated. Modifications of residues between the native sites of phosphorylation, such as to beneficially orient the phosphorylated residues to interact with hydroxyapatite or to reduce the distance between phosphorylation sites, is also contemplated. These modifications will either enhance or not significantly alter the structure, conformation or functional activity of the osteopontin or a functional fragment thereof.

Modifications that do not greatly affect the activity of osteopontin or its functional fragments can also include the addition or removal of sugar, phosphate or lipid groups as well as other chemical derivations known in the art. Additionally, osteopontin or its functional fragments can be modified by the addition of epitope tags or other sequences that aid in its purification and which do not greatly affect its activity.

As used herein, the term "functional fragment," in connection with osteopontin, is intended to mean a portion of osteopontin that maintains the ability of

osteopontin to inhibit ectopic calcification. A functional fragment can be, for example, from about 6 to about 300 amino acids in length, for example, from about 7 to about 150 amino acids in length, more preferably from about 8 to about 50 amino acids in length. If desired, a functional fragment can include regions of osteopontin with activities that beneficially cooperate with the ability to inhibit ectopic calcification. For example, a functional fragment of osteopontin can include sequences that promote the ingrowth of cells, such as endothelial cells and macrophages, at the site of ectopic calcification. Similarly, a functional fragment of osteopontin can include sequences, such as the RGD-containing domain, that beneficially promote cell adhesion and survival at the site of ectopic calcification.

As used herein, the term "individual" is intended to mean a human or other mammal, exhibiting, or at risk of developing, ectopic calcification. Such an individual can have, or be at risk of developing, for example, ectopic calcification associated with conditions such as atherosclerosis, stenosis, restenosis, renal failure, diabetes, prosthesis implantation, tissue injury or age-related vascular disease. The prognostic and clinical indications of these conditions are known in the art. An individual treated by a method of the invention can also be a candidate for, or have undergone, vascular surgery, including prosthetic valve replacement or angioplasty. An individual treated by a method of the invention can have a systemic mineral imbalance associated with, for example, diabetes, renal failure or kidney dialysis.

As used herein, the term "substantially the amino acid sequence," in reference to an osteopontin amino acid sequence or functional fragment thereof is intended to mean a sequence that is recognizably homologous to an osteopontin amino acid sequence and that inhibits ectopic calcification. For example, a sequence that is substantially the same as an osteopontin sequence can have greater than about 70% homology with an osteopontin sequence, preferably greater than about 80% homology, more preferably greater than about 90% homology.

As used herein, the term "prosthetic device" refers to a synthetic or biologically derived substitute for a diseased, defective or missing part of the body. As used herein, the term "bioprosthetic device" refers to a partially or completely biologically derived prosthetic device. Prosthetic devices are susceptible to ectopic calcification leading to premature failure, which can be inhibited by the methods of the invention. A prosthetic device can be implanted or attached at various sites of the body including, for example, the ear, eye, maxillofacial region, cranium, limbs and heart.

The methods of the invention can advantageously be used to prevent ectopic calcification of prosthetic heart valves, such as an aortic or atrioventricular valve, with or without a stent. Replacement heart valves can be made of a variety of materials, including metals, polymers and biological tissues, or any combination of these materials. Bioprosthetic valves include xenografted replacement valves from mammals, such as sheep, bovine and porcine, as well as human valves.

Bioprosthetic heart valves are commonly subjected to tissue fixation and can additionally be devitalized prior to implantation.

The invention provides a method of inhibiting ectopic calcification in an individual. The method consists of administering to the individual a therapeutically effective amount of osteopontin or a functional fragment thereof. The method is advantageous as it employs a molecule that normally occurs at the site of ectopic calcification as a therapeutic agent. Therefore, the method will result in minimal toxicity, immunogenicity and side effects.

Osteopontin can be prepared or obtained by methods known in the art including, for example, purification from an appropriate biological source or by chemical synthesis. An appropriate biological source of osteopontin can be tissues, biological fluids or cultured cells that contain or express osteopontin. The presence and abundance of osteopontin protein in a particular source can be determined, for example, using ELISA analysis (Min et al., Kidney Int. 53:189-93 (1998), incorporated herein by reference) or immunocytochemistry (O'Brien et al., Arterioscler. Thromb. 14:1648-1656 (1994), incorporated herein by reference).

Osteopontin has been determined to be present in or expressed by kidney cells, hypertrophic chondrocytes, odontoblasts, bone cells, bone marrow, inner ear and brain cells. Osteopontin is also found in biological fluids, including milk and urine. Osteopontin is also present in tumors, particularly metastatic tumors and is a component of kidney stones (Butler et al., In: Principles of Bone Biology, Bilezikian et al., eds.,

Academic Press, San Diego, pp. 167-181 (1996), incorporated herein by reference). Osteopontin is also produced by smooth muscle cells, macrophages and endothelial cells at the site of vascular lesions (O'Brien et al., Arterioscler. Thromb. 14:1648-1656 (1994), incorporated herein by reference). Therefore, osteopontin can be purified from any of these sources using biochemical purification methods known in the art.

Osteopontin can also be obtained from the secreted medium of cells of any of the above tissue lineages grown in culture. For example, osteopontin can be substantially purified from the conditioned medium of smooth muscle cell cultures as described by Liaw et al., Circ. Res. 74:214-224 (1994), incorporated herein by reference.

The nucleotide sequences of osteopontin from a variety of species are known, as described previously. Therefore, osteopontin or its functional fragments can also be recombinantly expressed by appropriate host cells including, for example, bacterial, yeast, amphibian, avian and mammalian cells, using methods known in the art. Methods for recombinant expression and purification of peptides in various host organisms are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainview, New York (1989) and in Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley and Sons, New York (1999), both of which are incorporated herein by reference. Methods for the recombinant synthesis and purification of osteopontin and exemplary functional fragments therefrom are described, for example, in Smith et al., J. Biol. Chem. 271:28485-28491 (1996), incorporated herein by reference.

Following recombinant synthesis and purification, osteopontin and its functional fragments can be modified in a physiologically relevant manner by, for example, phosphorylation, acylation or glycosylation, using enzymatic methods known in the art. A kinase that can be used to phosphorylate osteopontin or its functional fragments at biologically relevant sites is casein kinase II, as described in Example IV. Other serine-threonine kinases known in the art, such as protein kinase C can also be used to phosphorylate osteopontin or its functional fragments.

The methods of the invention can be practiced using osteopontin or any of its functional fragments that possess the activity of inhibiting ectopic calcification. Fragments of osteopontin are selected, produced by methods known in the art and screened as described herein to determine their ability to inhibit ectopic calcification.

Fragments of osteopontin can be produced, for example, by enzymatic or chemical cleavage of osteopontin. Methods for enzymatic and chemical cleavage and for purification of the resultant protein fragments are well known in the art (see, for example, Deutscher, Methods in Enzymology, Vol. 182, "Guide to Protein Purification," San Diego: Academic Press, Inc. (1990), which is incorporated herein by reference). As an example, osteopontin contains a thrombin cleavage site between Arg169 and Ser170. Either the N-terminal cleavage fragment or the C-terminal cleavage fragment of osteopontin can be used in the methods of the invention.

Fragments of osteopontin can also be produced by chemical or recombinant synthesis of peptides that

have substantially the sequence of osteopontin. For example, peptide libraries spanning overlapping sequences of osteopontin can be produced using methods known in the art and screened for their functional activity as described herein. Additionally, fragments corresponding to the N-terminal thrombin cleavage fragment or the C-terminal thrombin cleavage fragment of osteopontin can be recombinantly produced, as described by Smith et al., supra 271:28485-28491 (1996) and used in the methods of the invention.

As disclosed herein, osteopontin can inhibit ectopic calcification by directly adsorbing to and inhibiting apatite crystal growth and formation. Therefore, functional fragments of osteopontin can be selected based on their predicted ability to bind to calcium or calcium deposits. Regions that are contemplated to bind calcium include the aspartic acid rich sequence and the calcium binding homology domain. Therefore, a functional fragment of osteopontin can include, for example, substantially the sequence of the aspartic-rich calcium binding domain, DDMDDEDDDD (SEQ ID NO:3) or include substantially the sequence of the calcium binding homology domain, DWDSRGKDSYET (SEQ ID NO:4).

Additionally, as disclosed herein, phosphorylation can regulate the ability of osteopontin to inhibit ectopic calcification. Therefore, functional fragments of osteopontin can be selected by the presence of phosphorylation consensus sequences. A functional fragment of osteopontin can to chosen to include, for example, substantially the sequence of the casein kinase II phosphorylation consensus region, SGSSEEK (SEQ ID NO:5), or the C-terminal heparin binding homology domain

SKEEDKHLKFRISHELDSASSEVN (SEQ ID NO:6), which contains three conserved sites of serine phosphorylation. A functional fragment of osteopontin can alternatively or additionally include the alternatively spliced domain, NAVSSEETNDFKQE (SEQ ID NO:7), which contains two sites of serine phosphorylation. Additional sites of serine and threonine phosphorylation are described, for example, by Sorensen et al., Bioc. Biophys. Res. Comm. 198:200-205 (1994), incorporated herein by reference. A functional fragment of osteopontin can include one or several of these phosphorylated residues together with flanking amino acids.

Fragments of osteopontin having the ability to inhibit ectopic activity include regions of the molecule that are highly conserved among species. Regions within human osteopontin with high sequence conservation are presented, for example, in Giachelli et al., supra (1995). For example, a functional fragment can include the highly conserved sequence SDESHHSDESDE (SEQ ID NO:8). A functional fragment of osteopontin can also include the conserved cell adhesion domain, DGRGDSVAYG (SEQ ID NO:9) or the heparin binding homology domain RKKRSKKFRR (SEQ ID NO:10).

If desired, such as to optimize their functional activity, selectivity, stability or bioavailability, osteopontin or a functional fragment thereof can be modified to include D-stereoisomers, non-naturally occurring amino acids, and amino acid analogs and mimetics. Examples of modified amino acids are presented in Sawyer, Peptide Based Drug Design, ACS, Washington (1995) and Gross and Meienhofer,

The Peptides: Analysis, Synthesis, Biology, Academic Press, Inc., New York (1983), both of which are incorporated herein by reference.

If desired, one or more phosphorylated serine or threonine residues can be substituted by negatively charged amino acids, such as glutamic acid or aspartic acid. Such a modification can be advantageously made to reduce the susceptibility of osteopontin or a functional fragment to inactivation by phosphatases.

As disclosed herein, in addition to inhibiting ectopic calcification by directly adsorbing to and inhibiting apatite crystal growth and formation, osteopontin can also act by mediating an anti-calcific cellular response by promoting the accumulation and activation of carbonic anhydrase II (CAII) expressing multinucleated giant cells and macrophages, which are capable of acidifying the extracellular microenvironment and dissolving mineralized deposits. Through regulating CAII expression by multinucleated foreign body giant cells (FBGC) and macrophages, osteopontin can stimulate removal of calcium phosphate deposits.

The ability of osteopontin or a fragment selected and prepared as described above to inhibit ectopic calcification can be assayed by a variety of *in vitro* and *in vivo* assays known in the art or described herein. For example, as described in Example I, cultured vascular cells, such as bovine aortic smooth muscle cells, form calcified deposits in a time-dependent manner when treated with calcification medium containing β-glycerophosphate. Additionally, as described in Example III, human vascular smooth muscle cells form calcified deposits in the presence of elevated levels of

inorganic phosphate. Other culture systems for assaying the efficacy of osteopontin or a functional fragment thereof in inhibiting ectopic calcification can be determined by those skilled in the art. For example, osteopontin can be assayed using cells or tissues derived from other sites in the body where ectopic calcification occurs including, for example, viscera, skin, and endothelial cells.

The amount or extent of calcification prior to and following administering osteopontin or a functional fragment can be determined using such culture systems, either qualitatively by a visual or histochemical assessment, or by more quantitative methods. For example, calcified deposits can be detected visually as opaque areas by light microscopy, as black areas by von Kossa staining and as red areas by Alizarin Red S staining. The amount or extent of calcification can also be quantitatively assessed by the method described by Jono et al., Arterioscler. Thromb. Vasc. Biol. 17:1135-1142 (1997), incorporated herein by reference, or by using a commercially available colorimetric kit such as the Calcium Kit available from Sigma. Alternatively, the amount or extent of calcification can also be quantitatively assessed using known methods of atomic absorption spectroscopy.

As described in Examples I and III, the calcified deposits observed in cultured vascular smooth muscle cells, as assessed by histochemical, ultrastructural and electron diffraction analysis, can resemble the apatite deposits present at sites of ectopic calcification. Therefore, the ability of osteopontin or a functional fragment thereof to inhibit the deposition of calcium by cultured cells, in comparison with a

vehicle or protein control, is an accurate indicator of its ability to inhibit ectopic calcification in an individual.

The ability of osteopontin or a functional fragment thereof to inhibit ectopic calcification can also be tested in animal models known in the art to be reliable indicators of the corresponding human pathology. For example, ectopic calcification can be induced by the subcutaneous or circulatory implantation of bioprosthetic valves, such as porcine, sheep or bovine valves, into animals as described, for example, in Vyavahare et al., supra (1997). A reduction in the amount or rate of valve calcification by administration of osteopontin or a functional fragment can be detected, and is a measure of the functional activity of the preparation.

Similarly, animal models that are reliable indicators of human atherosclerosis, renal failure, hyperphosphatemia, diabetes, age-related vascular calcification and other conditions associated with ectopic calcification are known in the art. For example, topical and systemic calciphylaxis, calcinosis and calcergy, which are experimental models of ectopic calcification are described, for example, in Bargmann, J. Rheumatology 22:5-6 (1995), Lian et al., Calcified Tissue International, 35:555-561 (1983) and Boivin et al., Cell and Tissue Res. 247:525-532 (1987). An experimental model of calcification of the vessel wall is described, for example, by Yamaguchi et al., Exp. Path. 25:185-190 (1984).

A preferred animal model for examining ectopic calcification and the effect of osteopontin preparations is an osteopontin-deficient mouse, described by Liaw et

al., J. Clin. Invest. 101:1468-1478 (1998), incorporated herein by reference, in which, as described in Example V, ectopic calcification is enhanced compared to wild-type control animals.

Medical imaging techniques known in the art, such as magnetic resonance imaging, X-ray imaging, computed tomography and ultrasonography, can be used to assess the efficacy of osteopontin or a functional fragment thereof in inhibiting ectopic calcification in either a human or an animal. For example, the presence and extent of calcium deposits within vessels can be determined by the intravascular ultrasound imaging method described by Fitzgerald et al., Circulation 86:64-70 (1994), incorporated herein by reference. A decrease in the amount or extent of ectopic calcification can readily be identified and is indicative of the therapeutic efficacy of osteopontin or a functional fragment thereof.

Osteopontin or its functional fragments, assayed for their functional activity as described above, are administered to an individual in a therapeutically effective amount to inhibit ectopic calcification. Appropriate formulations, dosages and routes of delivery for administering osteopontin or a functional fragment are well known to those skilled in the art and can be determined for human patients, for example, from animal models as described previously. The dosage of osteopontin or a functional fragment thereof required to be therapeutically effective can depend, for example, on such factors as the extent of calcification, the site of calcification, the route and form of administration, the bio-active half-life of the molecule being administered, the weight and condition of the individual, and previous or concurrent therapies. The appropriate amount

considered to be a therapeutically effective dose for a particular application of the method can be determined by those skilled in the art, using the guidance provided herein. One skilled in the art will recognize that the condition of the patient needs to be monitored throughout the course of therapy and that the amount of the composition that is administered can be adjusted accordingly.

For treating humans, a therapeutically effective amount of osteopontin or its functional fragments can be, for example, between about 10 µg/kg to 500 mg/kg body weight, for example, between about 0.1 mg/kg to 100 mg/kg, or between about 1 mg/kg to 50 mg/kg, depending on the treatment regimen. For example, if osteopontin or a functional fragment is administered several times a day, or once a day, or once every several days, a lower dose would be needed than if osteopontin or a functional fragment were administered only once, or once a week, or once every several weeks. Similarly, formulations that allow for timed-release of osteopontin would provide for the continuous release of a smaller amount of osteopontin than would be administered as a single bolus dose.

Osteopontin or a functional fragment can be delivered systemically, such as intravenously or intraarterially, to inhibit ectopic calcification throughout the body. Osteopontin or a functional fragment can also be administered locally at a site known to contain or predicted to develop ectopic calcification. Such a site can be, for example, an atherosclerotic plaque, a segment of artery undergoing angioplasty or the site of prosthetic implantation. Appropriate sites for administration of osteopontin and its functional

fragments can be determined by those skilled in the art depending on the clinical indications of the individual being treated and whether or not the individual is concurrently undergoing invasive surgery.

Administration of osteopontin or a functional fragment can be achieved using various formulations of osteopontin. If desired, osteopontin can be administered as a solution or suspension together with a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be, for example, water, sodium phosphate buffer, phosphate buffered saline, normal saline or Ringer's solution or other physiologically buffered saline, or other solvent or vehicle such as a glycol, glycerol, an oil such as olive oil or an injectable organic ester.

A pharmaceutically acceptable carrier can additionally contain physiologically acceptable compounds that act, for example, to stabilize or increase the absorption of osteopontin or a functional fragment. Such physiologically acceptable compounds include, for example, carbohydrates such as glucose, sucrose or dextrans; antioxidants such as ascorbic acid or glutathione; chelating agents such as EDTA, which disrupts microbial membranes; divalent metal ions such as calcium or magnesium; low molecular weight proteins; lipids or liposomes; or other stabilizers or excipients. Osteopontin can also be formulated with a material such as a biodegradable polymer or a micropump that provides for controlled slow release of the molecule. Additionally, osteopontin can be formulated with a molecule, such as a phosphatase inhibitor, that reduces or inhibits dephosphorylation of osteopontin.

Osteopontin or a functional fragment can also be expressed from cells that have been genetically modified to express the protein. Expression of osteopontin from a genetically modified cell provides the advantage that sustained localized or systemic expression of the protein can occur, thus obviating the need for repeated administrations.

Methods for recombinantly expressing proteins in a variety of mammalian cells for therapeutic purposes are known in the art and are described, for example, in Lee et al., Transfusion Medicine II 9:91-113 (1995), which is incorporated herein by reference. Types of cells that are particularly amenable to genetic manipulation include, for example, hematopoietic stem cells, hepatocytes, vascular endothelial cells, keratinocytes, myoblasts, fibroblasts and lymphocytes.

A nucleic acid encoding osteopontin or a functional fragment can be operatively linked to a promoter sequence, which can provide constitutive or, if desired, inducible expression of appropriate levels of the encoded protein. Suitable promoter sequences for a particular application of the method can be determined by those skilled in the art and will depend, for example, on the cell type and the desired osteopontin expression level.

The nucleic acid encoding osteopontin or a functional fragment thereof can be inserted into a mammalian expression vector and introduced into cells by a variety of methods known in the art (see, for example, Sambrook et al., supra (1989); and Ausubel et al., supra (1994)). Such methods include, for example, transfection, lipofection, electroporation and infection

with recombinant vectors. Infection with viral vectors such as retrovirus, adenovirus or adenovirus-associated vectors is particularly useful for genetically modifying a cell. A nucleic acid molecule also can be introduced into a cell using known methods that do not require the initial introduction of the nucleic acid sequence into a vector.

In one embodiment of the invention, a prosthetic device can be contacted with osteopontin or a functional fragment thereof. Contacting a prosthetic device with osteopontin or a functional fragment will effectively prevent or reduce ectopic calcification of the prosthetic device, preventing failure of the device and the need for premature replacement. The prosthetic device can be contacted with osteopontin or a functional fragment either prior to, during or following implantation into an individual, as needed.

Osteopontin or a functional fragment can contact a prosthetic device by attaching the molecule either covalently or non-covalently to the prosthetic device. An appropriate attachment method for a particular application of the method can be determined by those skilled in the art. Those skilled in the art know that an appropriate attachment method is compatible with implantation of the prosthetic device in humans and, accordingly, will not cause unacceptable toxicity or immunological rejection. Additionally, an appropriate attachment method will enhance or not significantly reduce the ability of osteopontin or a functional fragment thereof to inhibit ectopic calcification of the prosthetic device and the surrounding tissue.

Methods for covalently attaching proteins to polymers, metals and tissues are known in the art. For example, osteopontin can be attached to the prosthetic device using chemical cross-linking. Chemical cross-linking agents include, for example, glutaraldehyde and other aldehydes. Cross-linking agents that link osteopontin or a functional fragment thereof to a prosthetic device through either a reactive amino acid group, a carbohydrate moiety, or an added synthetic moiety are known in the art. Such agents and methods are described, for example, in Hermason, Bioconjugate Techniques, Academic Press, San Diego (1996), which is incorporated herein by reference. These methods can be used to contact a prosthetic device with a therapeutically effective amount of osteopontin or a functional fragment thereof.

Osteopontin can also be attached non-covalently to the prosthetic device by, for example, adsorption to the surface of the prosthetic device. A solution or suspension containing osteopontin or a functional fragment thereof, together with a pharmaceutically acceptable carrier, if desired, can be coated onto the prosthetic device in a therapeutically effective amount.

To provide sustained delivery of osteopontin or a functional fragment, a prosthetic device can also be contacted with osteopontin or a functional fragment thereof produced by cells attached to the prosthetic device. Such cells can be seeded onto the prosthetic device and expanded either *ex vivo* or *in vivo*. Appropriate cells include cells that normally produce and secrete osteopontin including, for example, macrophages, smooth muscle cells or endothelial cells. Additionally, cells that have been genetically modified to produce

osteopontin or a functional fragment thereof including, for example, endothelial cells and fibroblasts, can be attached to the prosthetic device. The cells that are attached to the prosthetic device are preferably either derived from the individual receiving the prosthetic implant, or from an immunologically matched individual to reduce the likelihood of rejection of the implant.

The ability of osteopontin or a functional fragment that contacts a prosthetic device to inhibit ectopic calcification can be determined by various methods known in the art. One such method is to implant the prosthetic device into animals and measure calcium deposition, as described in Example V, in response to administration of osteopontin or a functional fragment thereof. Either a decrease in the rate or the amount of calcium deposition at the site of the explant is indicative of the therapeutic efficacy of the composition.

The invention provides a method of treating or inhibiting ectopic calcification by administering to an individual cells that acidify the local microenvironment, whereby the cells are targeted to the site of ectopic calcification. Multinucleate foreign body giant cells and macrophages are examples of cells capable of acidifying the local microenvironment. Therefore, the invention provides a method of treating or inhibiting ectopic calcification by administering to an individual macrophages, whereby the macrophages are targeted to the site of ectopic calcification. In addition, the invention provides a method of treating or inhibiting ectopic calcification by administering to an individual multinucleate foreign body giant cells, whereby the

multinucleate foreign body giant cells are targeted to the site of ectopic calcification.

As used herein, the term "local microenvironment" refers to the extracellular space that can be acidified either by recruitment and/or differentiation of acid producing cells or by promotion of proton production and extracellular release of cell protons. For example, osteoclasts and macrophages express carbonic anhydrase II, an enzyme that promotes the hydrolysis of carbon dioxide to carbonic acid. Carbonic acid serves as an intracellular source of protons that are exported from the cell via a vacuolar H^+-ATPase, thereby reducing local pH and promoting mineral dissolution. Therefore, the invention provides a method of treating or inhibiting ectopic calcification via osteopontin-mediated expression of carbonic anhydrase II by macrophages and multinucleate foreign body giant cells.

As disclosed herein, osteopontin regulates ectopic mineralization not only by directly binding to apatite crystal surfaces and inhibiting crystal growth (see Example II), but also indirectly by regulating mineral loss (Example V). Specifically, osteopontin acts as a mediator of an anti-calcific cellular response by promoting macrophage recruitment, increased carbonic anhydrase II expression and extracellular acidification.

The cells capable of acidifying the local microenvironment such as macrophages and multinucleate foreign body giant cells are targeted to the site of ectopic calcification. For example, macrophages and multinucleate foreign body giant cells can be attached to the prosthetic device. For example, macrophages and

multinucleate foreign body giant cells can be seeded onto the bioprosthetic heart valve and expanded *ex vivo* or *in vivo* as described above. Additionally, cells that have been genetically modified to express carbonic anhydrase II, or a functional fragment thereof, can be attached to the prosthetic device. A functional fragment of carbonic anhydrase II retains osteopontin-mediated regulation as well as catalytic activity. As described above for osteopontin-producing cells, the carbonic anhydrase II-producing cells are preferably derived from the individual receiving the prosthetic implant or from an immunologically matched individual to reduce the likelihood of implant rejection. In addition, macrophages and multinucleate foreign body giant cells can be administered to the site of ectopic calcification in an individual either directly or systemically. If administered systemically, the administered macrophages and multinucleate foreign body giant cells can be targeted to the site of ectopic calcification. For example, precoating a bioprosthetic device with osteopontin or osteopontin antibody is one way to target systemically administered macrophages and multinucleate foreign body giant cells to a site of ectopic calcification to acidify the local microenvironment.

As described in Example V, acidification of the local microenvironment can also be achieved by administering osteopontin, which can regulate carbonic anhydrase II expression by macrophages and multinucleate foreign body giant cells. Methods of administering osteopontin to an individual are described in detail above. The expression level of osteopontin required to stimulate carbonic anhydrase II expression can be significantly lower than the expression level required to directly inhibit hydroxyapatite growth. Therefore, a

therapeutically effective amount for stimulating carbonic anhydrase II can be within the range of about $1x10^{-12}$ M to $5x10^{-7}$ M . Therapeutically effective amounts of osteopontin sufficient to stimulate expression of carbonic anhydrase II can also be within the range of about $1x10^{-12}$ M to $1x10^{-7}$ M, $1x10^{-12}$ M to $5x10^{-8}$ M, $1x10^{-12}$ M to $1x10^{-8}$ M, $1x10^{-12}$ M to $5x10^{-9}$ M, $1x10^{-12}$ M to $1x10^{-9}$ M, $1x10^{-12}$ M to $5x10^{-10}$ M, $1x10^{-12}$ M to $5x10^{-10}$ M, or $1x10^{-12}$ M to $5x10^{-11}$ M.

Thus, the invention provides a method of treating or inhibiting ectopic calcification by administering to an individual cells that acidify the local microenvironment. The invention further provides a method of treating or inhibiting ectopic calcification in an individual comprising promoting recruitment of acid producing cells to a site of ectopic calcification by administering osteopontin. The invention also provides a method of treating or inhibiting ectopic calcification in an individual comprising promoting differentiation of acid producing cells at a site of ectopic calcification by administering osteopontin. The invention further provides a method of treating or inhibiting ectopic calcification in an individual comprising promoting recruitment and differentiation of acid producing cells to a site of ectopic calcification by administering osteopontin. As described in Experiment V, mice carrying one copy of the osteopontin gene express high enough levels of osteopontin to promote formation of carbonic anhydrase II positive cells in response to ectopic mineralization (see Figure 13). Macrophages and multinucleate foreign body giant cells are carbonic anhydrase II-expressing cells derived from a similar hematopoietic origin and shown herein to be localized

adjacent to mineralized implants in mice carrying one copy of the osteopontin gene.

As described in Example V, osteopontin can control mineral resorption by recruiting acid producing cells to the implantation site of gutaraldehyde-fixed aortic valve (GFAV) leaflets in mice. For example, as demonstrated by immunostaining, osteopontin can mediate the recruitment of macrophages and promote CAII-positive cell formation (see Example V). As disclosed herein, macrophages and multinucleate foreign body giant cells are examples of cells expressing carbonic anhydrase II that are regulated by osteopontin. Methods of administering osteopontin are described in detail above.

The invention also provides a method of treating or inhibiting ectopic calcification in an individual by increasing the expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin. As disclosed herein, osteopontin controls mineral resorption by regulating the differentiation and activity of carbonic anhydrase II expressing cells, including macrophages and multinucleate foreign body giant cells (see Example V).

It is understood that modifications that do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Calcification of cultured bovine vascular cells

This example demonstrates that calcium deposition by cultured bovine aortic smooth muscle cells is a credible model of ectopic calcification. Methods for inducing physiologically relevant calcification are described. These methods can be used to assay preparations of osteopontin and fragments thereof for their ability to inhibit ectopic calcification.

Culture of bovine aortic smooth muscle cells

BASMCs were obtained by a modification of the explant method originally described by Ross et al., J. Cell Biol., 50:172-186 (1971), which is incorporated herein by reference. Briefly, medial tissue was separated from segments of bovine thoracic aorta. Small pieces of tissue (1 to 2 mm^3) were loosened by a one-hour incubation in DMEM containing 4.5 g/L of glucose supplemented with 165 U/ml collagenase type I, 15 U/ml elastase type III and 0.375 mg/mL soybean trypsin inhibitor at 37°C. Partially digested tissues were placed in 6-well plates and cultured for several weeks in DMEM containing 4.5 g/L of glucose supplemented with 20% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells that had migrated from the explants were collected and maintained in growth medium (DMEM containing 15% FBS and 10 mM sodium pyruvate supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin). To confirm that the cells isolated from bovine aortic wall were

vascular smooth muscle cells, α-smooth muscle actin, vimentin, and calponin levels were examined by immunofluorescence microscopy.

For immunofluorescence microscopy, BASMCS were cultured on 10-well heavy Teflon-coated microscope glass slides (Cel-Line Associates Inc., USA) for 24 hours, fixed with cold methanol, blocked with PBS containing 2% BSA and 10% normal rabbit serum, and treated with monoclonal anti-α-smooth muscle actin antibody (1A4, Sigma) and monoclonal anti-vimentin antibody (V9, Dako) diluted with PBS containing 2% BSA 1:50 and 1:25, respectively. Monoclonal anti-calponin antibody (CALP), Frid et al., Dev. Biol., 153:185-193 (1992), was used without dilution. As a secondary antibody, FITC-conjugated rabbit anti-mouse IgG was used after dilution with PBS 1:30. Mouse non-immune IgG was used as a control for the primary antibody.

Greater than 95% of the cells obtained as described above were stained with α-smooth muscle actin, vimentin, and calponin antibodies in a filamentous pattern, indicating that the cultured cells were of vascular smooth muscle origin. For all experiments, cells were used between passages 2 and 5.

Calcium deposition by bovine aortic smooth muscle cells

In order to examine calcification by cultured BASMC smooth muscle cells, calcification was induced by the method described by Shioi et al., Arterioscler Thromb Vasc Biol., 15:2003-2009 (1995), which is incorporated herein by reference. Briefly, BASMC were cultured in growth medium for 4 days, and then switched to calcification medium (DMEM (high glucose, 4.5 g/L)

containing 15% FBS and 10 mM sodium pyruvate in the presence of 10 mM of β-glycerophosphate (unless otherwise stated), 10-7 M insulin, and 50 µg/ml of ascorbic acid, supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin) for 10 days. The medium was replaced with fresh medium twice a week. In the time course experiments, the beginning day of culture in calcification medium was defined as day O.

Calcification was assessed by a modification of the method described by Jono et al., Arterioscler. Thromb. Vasc. Biol. 17:1135-1142 (1997) which is incorporated herein by reference. Briefly, the cultures were decalcified with 0.6 N HCl for 24 hours. The calcium content of the HCl supernatant was determined colorimetrically by the o- cresolphthalein complexone method (Calcium Kit, Sigma). After decalcification, the cultures were washed with phosphate-buffered saline (PBS) and solubilized with 0.1 N NaOH/O. 1% sodium dodecyl sulfate(SDS). Total protein content was measured with a Bio-Rad Protein Assay Kit (Bio-Rad). The calcium content of the cell layer was normalized to protein content. Phosphorus and calcium concentrations in the culture medium were measured by the phosphomolybdate complex method (Phosphorus Kit, Sigma) and the o-cresolphthalein complexone method (Calcium Kit, Sigma), respectively. Values were expressed as the mean +/- SEM, n=3.

BASMC treated with calcification medium containing β-glycerophosphate initiated calcium-containing mineral deposition in a time-dependent manner over the course of 14 days. In contrast, BASMC cultured in growth medium lacking β-glycerophosphate did not calcify. Addition of β-glycerophosphate resulted in an

increased phosphorus concentration which correlated positively with calcium deposition in the cell layer. Conversely, calcium concentration decreased in the culture medium as the cell layer became calcified.

The effects of β-glycerophosphate on calcium deposition, phosphorus concentration and calcium concentration in the medium were dose-dependent. Calcium deposition depended on the initial concentration of β-glycerophosphate and was half-maximal at ~4 mM β-glycerophosphate. Phosphorus concentration in the culture medium increased with increasing concentrations of β-glycerophosphate over the range of 0 to 10 mM. Calcium deposition in the culture medium was inversely proportional to calcium deposition in the cell layer.

The observed calcification was not due to spontaneous precipitation of mineral from the media as supplementation of the culture media with up to 10 mM inorganic phosphate failed to form calcified deposits in the absence of cells. Nor did addition of calcification media to endothelial cell cultures induce mineralization.

These results indicate that the calcification of BASMC under conditions which elevate inorganic phosphate in the media is a specific, cell- and matrix-mediated event.

Morphology of BASMC calcification

To determine whether the calcification process in the BASMC cultures represented a physiologic-type of mineralization, histochemical, ultrastructural, and electron diffraction analyses were performed.

Mineral deposition by BASMC cultures was assessed histochemically by von Kossa staining (30 minutes, 5% silver nitrate) and light microscopy using the method described by Mallory, F.B., in Pathological Techniques, Second Edition, Philadelphia, WB Saunders Co., p. 152 (1942), which is incorporated herein by reference). The expression of alkalinephosphatasee was visualized by incubating citrate-acetone-formaldehyde fixed cells at room temperature for 15 minutes with Naphthol AS-BI Alkaline Solution (Sigma).

For ultrastructural examination by transmission electron microscopy (TEM), BASMC cells grown on plastic were fixed overnight in an aldehyde solution containing 1% glutaraldehyde and 1% paraformaldehyde buffered with 0.1 M sodium cacodylate buffer at pH 7.2. The cultures were then washed with 0.1 M sodium cacodylate buffer alone, dehydrated in a graded series of ethanol solutions, and infiltrated and embedded in either Taab epoxy resin or LR White acrylic resin (Marivac, Nova Scotia, Canada). The resins were polymerized for 2 days at 55°C. Samples destined for epoxy embedding were also post-fixed with potassium ferrocyanide-reduced 4% osmium tetroxide to provide additional membrane contrast in the electron microscope.

For mineral analyses by selected-area electron diffraction, other cultures were treated nonaqueously by fixing only with 100% ethanol, followed by direct embedding in resin without further processing. One micrometer-thick survey sections were prepared from various regions of the cultures and stained with Toluidine blue for examination by light microscopy. Thin sections (80-100 nm) of selected regions were then cut using a diamond knife on a Reichert Ultracut E microtome

and placed on Formvar-coated nickel grids evaporated with carbon. Grid-mounted sections were stained briefly with ethanolic uranyl acetate and lead citrate and examined using a JEOL JEM 1200EX transmission electron microscope operating at 60 kV. Anhydrously treated samples left unstained were used for selected-area electron diffraction using a 100 μM diffraction aperture and a camera length of 80 cm. Diffraction patterns were analyzed and compared with synthetic apatite standards and powder diffraction files as previously reported for bone mineral (Landis et al., J. Ultrastruc. Res., 63:188-223 (1978), incorporated herein by reference).

By light microscopy, BASMC cultures grown in growth medium showed areas of monolayer and multilayered growth typical for these types of cells. Following treatment with calcification medium for 10 days, the cultures showed extensive deposition of mineral, predominantly in multilayered areas. Von Kossa staining confirmed the presence of phosphate-containing mineral in these cultures. The calcification was most often observed in the extracellular matrix between cells, and was typically more pronounced at the basal aspect of the culture. The BASMCs in these calcified cultures were also positive for alkaline phosphatase activity.

At 14 days of culture (10 days with β-glycerophosphate), BASMC were monolayered or multilayered and at some locations formed nodules of cells. Ultrastructurally, where multilayered or nodular in appearance, the cells were associated with abundant extracellular matrix rich in collagen fibrils. At sites of this extracellular matrix accumulation, cells exhibited well-developed organelles typically associated with protein synthesis and secretion. A prominent

cytoskeleton was evidenced by an extensive network of intracellular microfilaments, most likely composed of actin.

Whereas cells cultured without β-glycerophosphate showed no evidence of extracellular matrix calcification, those cultured with the added organic phosphate source showed several morphologically distinct forms of calcification associated with the cell layer. These included roughly spherical aggregates of calcified collagen fibrils, nodular deposits with increased mineral density at the periphery, and more diffuse calcification involving both the intra- and interfibrillar compartments of the extracellular matrix. At these latter sites, crystals having somewhat larger dimensions were observed to extend from one collagen fibril to another. Membrane-bounded matrix vesicles were also found in the extracellular matrix. Selected-area electron diffraction of anhydrously treated and unstained tissue sections of BASMC cultures containing calcified deposits identified the mineral phase as apatite, showing prominent diffraction reflections (from lattice planes 002, 211, 112, 300) whose indices were characteristic for this type of mineral.

Alkaline phosphatase is required for normal bone mineralization (Whyte et al. Endocr. Rev., 15:439-461 (1994)) and has been shown to be required for calcification of osteoblast and cartilage cell cultures in response to β-glycerophosphate (Tenenbaum et al., Bone Mineral, 2:13-26 (1987)). To determine whether alkaline phosphatase was required for calcification in BASMCs under the conditions used in these studies, cultures were treated with the alkaline phosphatase inhibitor levamisole, or with vehicle alone. Calcium deposition in

BASMC cultures was dose-dependently inhibited by levamisole. Half-maximal inhibition was observed at 5 x 10^{-5} M levamisole. Vehicle treatment had no effect. Levamisole treatment was associated with a decrease in phosphorus concentration and maintenance of high calcium concentration in the culture medium.

These results indicate that calcification of the matrix deposited by BASMC cultures resembles the mineralization observed at sites of ectopic calcification in regard to mineral type (apatite) and the ultrastructure of the calcified deposits. For example, mineralization occurred predominantly in association with extracellular matrix collagen fibrils and matrix vesicles. Similar vesicular structures have been reported in calcified atherosclerotic plaques in association with elevated alkaline phosphatase activity (Kim et al., Fed Proc, 35:156-162 (1976)). Additionally, the nodular calcifications present in the calcifying BASMC cultures indicate spherulitic crystal growth which is a common observation in calcified atherosclerotic plaques and valves (Kim et al., Fed Proc, 35:156-162 (1976)).

Therefore, the calcifying BASMC cultures used in these studies are able to create an extracellular milieu capable of mineralization similar to the mineralization observed in calcified vascular tissues *in vivo*, supporting their use as a model of ectopic calcification.

EXAMPLE II

Osteopontin inhibits BASMC calcification

This example demonstrates that osteopontin inhibits BASMC calcification *in vitro*, which is a credible model of ectopic calcification *in vivo*. Therefore, osteopontin will be a therapeutically effective inhibitor of ectopic calcification.

Rat osteopontin was purified from the conditioned medium of rat neonatal smooth muscle cell cultures as described by Liaw et al., supra 74:214-224 (1994), which is incorporated herein by reference. This preparation was judged to be >95% pure based on Coomassie staining and N-terminal sequence analysis.

To examine the effect of osteopontin on BASMC-mediated calcification *in vitro*, soluble osteopontin or vehicle alone (0.1 mM sodium citrate) was added to the calcifying BASMC cultures. As shown in Figure 2a, osteopontin at 0.05, 0.5 and 5 µg/ml dose-dependently inhibited calcification assessed at 10 days. For example, 0.5 µg/ml of osteopontin inhibited calcification by approximately 90%, and 5 µg/ml osteopontin almost completely inhibited calcification. In contrast, vehicle alone had no effect (Figure 2a). Therefore, low concentrations of exogenously applied osteopontin profoundly inhibits extracellular mineralization in a calcifying vascular cell culture system.

To exclude the possibility that contaminants in the osteopontin preparation were responsible for the observed inhibitory effect, immunodepletion experiments were performed. Medium containing 10 µg/ml osteopontin was mixed with 20 mg/ml anti-osteopontin (OP-199) or normal goat IgG, prepared by the method described by Liaw et al., supra (1994) and incubated for 1 hr at room temperature. 250 mg protein-A-sepharose was added and incubated for 1 hr at room temperature. The antibody-protein A sepharose complexes were removed by centrifugation, and the remaining supernatant diluted twenty-fold for use in the calcification studies. Unpaired Student's t test was employed to compare groups and a probability value (p) value less than 0.05 was considered significant.

Medium containing 0.5 ug/ml rat osteopontin inhibited calcification of the cultures by 18 fold (5.05 ± 0.25 µmole/mg for vehicle-treated versus 0.33 ± 0.06 µmole/mg for osteopontin-treated BASMC p=0.0023). Immunodepletion of the osteopontin solution with osteopontin antibody significantly reduced its inhibitory activity (0.33 ± 0.06 µmole/mg for nonimmunodepleted sample versus 2.60 ± 0.43 µmole/mg for anti-osteopontin depleted samples, p=0.0338). In contrast, immunodepletion with normal goat IgG did not affect the inhibitory activity of the rat osteopontin solution (0.49 ± 0.10 µmole/mg for normal goat IgG-treated versus 0.33 ± 0.06 µmole/mg with no immunodepletion, p=0.2480).

These results confirm that the observed inhibition of BASMC-mediated calcification by the osteopontin preparation was specifically due to osteopontin, rather than due to a contaminant.

To determine the specificity and uniqueness of osteopontin's effects, two additional noncollagenous extracellular matrix RGD-containing molecules with limited structural and functional homology to osteopontin, vitronectin and fibronectin, were tested for their ability to inhibit BASMC-mediated calcification. Rat plasma vitronectin (Sigma Immunochemicals, USA) and bovine fibronectin (TELIOS Pharmaceutical Inc., USA) were resuspended in PBS at a concentration of 0.5 mg/ml and stored frozen until use. As shown in Figure 2b, vitronectin (VN) and fibronectin (FN), at equimolar concentrations as were effective for osteopontin, were unable to inhibit calcium deposition. Therefore, the effect of osteopontin on inhibiting vascular calcification is highly specific. Furthermore, these results indicate that the capacity of osteopontin to modulate mineralization are unrelated to its RGD-dependent cell adhesive functions.

Mechanism of osteopontin inhibition

The mechanism by which osteopontin inhibited calcification was tested. One possibility was that osteopontin might function in a manner similar to levamisole by affecting alkaline phosphatase activity, thereby inhibiting production of inorganic phosphate from β-glycerophosphate and preventing calcium phosphate deposition.

For cellular alkaline phosphatase activity measurements, cells were cultured in calcification medium in the presence of various concentrations of osteopontin. Cells were washed three times with PBS and cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for

alkaline phosphatase activity by the method described by Bessey et al., J. Biol. Chem. 164:321-329 (1946), which is incorporated herein by reference. One unit was defined as the activity producing 1 nmol of p-nitrophenol within 1 minute. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad). The data were normalized to the protein content of the cell layer.

Treatment with osteopontin did not affect the alkaline phosphatase activity of BASMC cultures, as shown in Figures 3a and 3c. The addition of osteopontin also did not reduce the phosphorus concentration of the medium. In contrast, levamisole dose-dependently inhibited BASMC alkaline phosphatase activity (Figure 3b) and reduced the phosphorus concentration in the culture medium. These results demonstrate that osteopontin does not act by inhibiting alkaline phosphatase activity.

The possibility that osteopontin chelates or sequesters calcium in the culture media to prevent mineralization was also tested. The initial calcification medium was supplemented with increasing concentrations of calcium in the presence of osteopontin or vehicle alone. Cultures were then allowed to calcify in the presence or absence of osteopontin over a 10 day period. As shown in Figure 4a, increasing the calcium content of the medium was able to overcome the inhibitory effect of osteopontin on calcium deposition, allowing more mineral to be deposited in the cell layer. Consistent with this, a decrease in the phosphorus content (from 8.2 mM to 7.3 mM) of the culture media was noted (Figure 4b).

The calcium content of the media at the end of the 10 day period in the presence of osteopontin was also

measured. If osteopontin acted by sequestering calcium, it was expected that either a constant or an increasing amount of calcium would be observed in the medium, reflecting retention of calcium in the medium by osteopontin binding. However, the opposite was observed. Calcium concentration in the culture medium was decreased at the end of the 10 day period compared to initial calcium concentrations, and correlated inversely with calcium deposition (compare Figures 4a and 4c). Therefore, the inhibitory effect of osteopontin on mineralization is calcium dependent (i.e. decreased by increasing calcium concentrations), but is not attributable to chelation of the calcium available in the medium. This observation is consistent with the known calcium binding properties of osteopontin. It has been shown that about 50 molecules of calcium can be bound by osteopontin at physiological calcium concentrations (Chen et al., J. Biol. Chem. 267:24871-24878 (1992)). Hence it would require about 40 μM osteopontin (2.7 mg/ml) to chelate 2 mM calcium, which is more than 5000 times the amount of osteopontin (0.5 μg/ml) demonstrated to be effective in inhibiting vascular calcification in the assays described herein.

The ultrastructural localization of endogenous and exogenous osteopontin in the BASMC cultures was also determined using immunogold labeling to further characterize the mechanism of osteopontin inhibition of vascular calcification. BASMC were cultured in calcification media for 7 days to allow mineralization to begin. Purified rat osteopontin (0.5 μg/ml) was then added until day 10. Cultures were preserved using aldehyde fixative followed by embedding in LR White acrylic resin for immunocytochemistry. Post-embedding immunolabeling was performed using osteopontin antibody

(OP-199) and protein A-colloidal gold complex as described by McKee et al., Microscop. Res. And Tech., 33:141-164 (1996), which is incorporated herein by reference. Briefly, thin (80 nm) sections of the cultures were placed on nickel grids and incubated for 5 min with 1% ovalbumin in PBS, followed by incubation with primary antibody for 1 hr, rinsing with PBS, blocking again with ovalbumin, and then exposure to protein A-gold complex for 30 min. After final rinsing with distilled water, grids were air dried and conventionally stained with uranyl acetate and lead citrate and viewed by transmission electron microscopy. The specificity of the OP- 199 antibody has been shown previously by Western blotting (Liaw et al., supra, (1994)) and by incubations using pre-immune serum and protein A-gold complex alone.

For these immunogold labeling studies, osteopontin was omitted (vehicle alone) or added on day 7 following initiation of mineralization with β-glycerophosphate. Under these conditions, exogenously applied osteopontin (0.5 ug/ml) was still able to inhibit BASMC culture calcification by 50% at day 10. A low level of endogenous osteopontin was found in untreated, mineralizing cultures, typically in a diffuse pattern in the mineralized areas. In contrast, in osteopontin-treated cultures, gold particles were abundant at sites of calcification, typically accumulating at the margins of small calcified masses or associating with individual crystal profiles. No gold particles were observed when pre-immune serum and protein A-gold complex alone were used as controls, indicating that a direct interaction of osteopontin with the growing apatite crystals is required for its inhibitory function. Osteopontin was not observed to be associated with unmineralized matrix or cells.

The results described above demonstrate that osteopontin is able to inhibit physiological calcification mediated by vascular cells at low concentrations by direct binding of osteopontin to apatite crystal surfaces and inhibition of crystal growth. Therefore, osteopontin will be therapeutically useful in preventing and treating ectopic calcification.

EXAMPLE III

Calcification of cultured human vascular cells

This example shows that calcium deposition by cultured human smooth muscle cells in the presence of elevated inorganic phosphate is a credible model of ectopic calcification. Methods for inducing physiologically relevant calcification are described. These methods can be used to assay preparations of osteopontin and fragments thereof for their ability to inhibit ectopic calcification.

The normal adult range of serum inorganic phosphate concentration is about 1.0-1.5 mM. A high serum phosphate level, or hyperphosphatemia, occurs in association with various disease states including, for example, chronic renal failure and subsequent kidney dialysis. In such disease states the serum inorganic phosphate levels can typically exceed 2 mM. In order to model ectopic calcification associated with hyperphosphatemia and to determine the effect of osteopontin and its functional fragments on such calcification, a relevant *in vitro* model system for calcification was developed, as follows.

Human vascular smooth muscle cells (HSMC) were obtained by enzymatic digestion as described by Ross, J. Cell Biol. 50:172-186 (1971) and Liaw et al., J. Clin. Invest. 95:713-724 (1995), incorporated herein by reference. Briefly, medial tissues were separated from segments of human aorta obtained at heart transplant surgery and autopsy, respectively. For plaque SMC, coronary atherectomy-derived tissues were obtained at time of surgery. Small pieces of tissue (1 to 2 mm^3) were digested overnight in DMEM supplemented with 165 U/ml collagenase type I, 15 U/ml elastase type III and 0.375 mg/ml soybean trypsin inhibitor at 37°C. Single cell suspensions were placed in 6-well plates and cultured for several weeks in DMEM supplemented with 20% FCS at 37°C in a humidified atmosphere containing 5% CO_2. Cultures that formed colonies were collected at confluence and maintained in growth medium (DMEM containing 15% FBS and 1 mM sodium pyruvate supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin; final inorganic phosphate concentration was 1.4 mM). Purity of cultures was assessed by positive immunostaining for alpha-SM actin and calponin and absence of von Willebrand factor staining as described by Ross, supra (1971) and Liaw et al., supra (1995).

Primary human adult and fetal aortic medial and coronary plaque primary cells up to passage 8 were used in these experiments. A fetal and adult HSMC culture was also immortalized using HPV-E6E7 and characterized as described by Perez et al., Proc. Natl. Acad. Sci. USA 89:1224-1228 (1992), incorporated herein by reference.

HSMC were routinely subcultured in growth medium. At confluence, cells were switched to calcification medium (DMEM containing 15% FBS and 1 mM

sodium pyruvate in the presence of 2 mM inorganic phosphate supplemented with 100 U/ml penicillin and 100 µg/ml of streptomycin) for up to 14 days. The medium was replaced with fresh medium every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0. Calcium deposition was quantified and assessed histochemically and cytochemically as described above in Example I.

In medium containing normal serum phosphate levels (inorganic phosphate, P_i, of 1.4 mM), HSMC accumulated very little calcium mineral. In contrast, in the presence of 2 mM inorganic phosphate, calcium deposition increased in a time-dependent manner. For example, on day 9, calcified HSMC vs. uncalcified control was 210.3 +/- 2.4 vs. 15.1 +/- 2.4 (µg/mg protein), mean +/- SEM (n+3)). The effect of inorganic phosphate on calcium deposition was dose-dependent over the range of 1.4 mM to 2 mM inorganic phosphate. Induction of calcification by elevated inorganic phosphate appeared to be a general feature of human cells, since primary HSMC derived from different sources (human adult and fetal aortic and coronary plaque) as well as immortalized derivatives of these cells showed similar behavior. No spontaneous deposition of calcium mineral occurred in calcification medium or in medium supplemented with up to 10 mM inorganic phosphate, indicating that cells and/or cell-derived matrix is necessary for mineralization.

To determine whether the observed calcification in the human cell culture system was physiologically relevant, morphological studies were performed. After culturing HSMC in calcification medium for 10 days, granular deposits developed throughout the cell culture. The deposits were identified as phosphate-containing

mineral by von Kossa staining, as described in Example I. Black-stained particles were diffusely scattered throughout the cell layer, predominantly in the extracellular regions, with greatest accumulation in multilayered foci. Electron microscopic analysis confirmed the presence of an apatite mineral phase, calcified collagen fibrils and matrix vesicles associated with mineralized cultures, essentially identical to the calcification of bovine SMC cultures in the system described in Example I.

These results demonstrate that HSMC cultures are susceptible to calcification when cultured in media containing inorganic phosphate concentrations typically found in hyperphosphatemic individuals. Furthermore, the observed calcification in the cultured human cells is similar to the ectopic calcification observed in calcified tissues *in vivo*. Therefore, the HSMC calcification culture system can be used to accurately assess the effect of regulators of ectopic calcification.

EXAMPLE IV

Inhibition of calcification of human vascular cells by osteopontin and functional fragments thereof

This example demonstrates that osteopontin and exemplary functional fragments of osteopontin effectively inhibit human smooth muscle cell calcification. Therefore, osteopontin can be used therapeutically to inhibit ectopic calcification.

Osteopontin proteins and functional fragments were assayed for their ability to inhibit ectopic calcification using the HSMC calcification system

described in Example III. The osteopontin proteins include full-length human recombinant osteopontin as well as recombinant N-terminal and C-terminal human osteopontin fragments similar to those that would be formed following thrombin cleavage of the native protein, as described by Smith et al., supra (1996). Two N-terminal fragments were used, 10N and 30N, which refer to two differ splice variants of osteopontin. The 30N splice variant contains an additional 14 amino acids, NAVSSEETNDFKQE (SEQ ID NO:7), which correspond to exon 5 (amino acids 59-72). The 10N fragment contains amino acids 17-58 and 73-160 of native osteopontin, whereas the 30N fragment contains amino acids 17-169. The 10C fragment contains the C-terminal domain of osteopontin, amino acids 170-317.

The N- and C-terminal recombinant osteopontin fragments were expressed as fusion proteins with GST, purified from bacterial lystates by affinity chromatography on glutathione beads, and cleaved with thrombin. The full-length human recombinant osteopontin was prepared as a His-tagged protein. The size and purity of the resulting recombinant proteins was confirmed by SDS-PAGE analysis (Smith et al., supra (1996)).

Recombinant osteopontin and its functional fragments were assayed for their ability to inhibit ectopic calcification of human smooth muscle cells (HSMC) either prior to or following phosphorylation by casein kinase II. The amount of phosphate incorporated into osteopontin (OPN) and its fragments achieved by casein kinase II phosphorylation is shown in Figure 5b. As shown in Figure 5a, in the presence of high-phosphate calcification medium, calcium deposition into HSMC matrix

is reduced to basal levels by the addition of phosphorylated OPN, 30N OPN, 10N OPN or 10C OPN. The non-phosphorylated forms of these proteins do not significantly affect calcium deposition in this assay. These results show that both N- and C-terminal fragments of osteopontin are functional fragments of osteopontin, and that serine-threonine phosphorylation appears to be important for the functional activity of osteopontin and its functional fragments.

As shown in Figure 6, recombinant osteopontin phosphorylated by casein kinase II is able to inhibit HSMC calcification at a concentration of 15 nM. Dephosphorylation with alkaline phosphatase (ALP) reverses this inhibitory ability. These results confirm the importance of phosphorylation for the functional activity of osteopontin and its functional fragments.

The effect of human osteopontin on inhibiting ectopic calcification is dose-dependent over the range of concentrations of 0.1 µg/ml to 5.0 µg/ml (Figure 7). Furthermore, the effect of osteopontin on inhibiting and reversing ectopic calcification is rapid, with significantly reduced calcium deposition being apparent by 60 minutes, with approximately 50% inhibition observable by 90 minutes following addition (Figure 8).

These results indicate that osteopontin and exemplary functional fragments thereof are able to effectively inhibit physiologically relevant ectopic calcification of human cells rapidly and at low concentrations. Therefore, full-length osteopontin and functional fragments thereof will be therapeutically

effective in inhibiting ectopic calcification in individuals exhibiting or at risk of exhibiting ectopic calcification.

EXAMPLE IV

Osteopontin inhibits ectopic calcification *in vivo*

This example shows that osteopontin inhibits ectopic calcification *in vivo*.

The effect of subcutaneous implantation of porcine prosthetic valves in normal mice and mice deficient in osteopontin was tested to determine the role of osteopontin in ectopic calcification *in vivo*. Mice deficient in one or both copies of the osteopontin gene are described in Liaw et al., supra (1998). A 4.0 mm^2 piece of porcine glutaraldehyde-fixed aortic valve leaflet was subcutaneously implanted into 5-6 week old female mice carrying either the wild type (WT), heterozygote (HTZ) or null allele (KO) for osteopontin. After 14 days, implants were removed, freeze-dried and acid hydrolyzed. Calcium levels were assayed as described in Example I and normalized to the dried weight of the explant.

As shown in Figure 9, implanted valves calcify to a significantly greater extent in osteopontin null mice than in wild-type or heterozygous mice. Therefore, consistent with the observed ability of osteopontin to inhibit ectopic calcification in relevant *in vitro* systems, these results indicate that osteopontin inhibits ectopic calcification *in vivo*.

The foreign body inflammatory response also appears to be impaired in the osteopontin null mouse. For example, there is an apparent reduction in infiltration by macrophages at the site of valve implantation in the osteopontin null mouse compared to the wild-type or heterozygous mice. Macrophages that normally infiltrate a site of inflammation and ectopic calcification are contemplated to promote removal of calcified deposits by phagocytosis. Therefore, it is contemplated that osteopontin both inhibits hydroxyapatite formation and promotes phagocytotic resorption of calcified deposits by macrophages.

Accordingly, the administration of osteopontin or its functional fragments to an individual will be therapeutically effective in inhibiting ectopic calcification.

Example V

Osteopontin-mediated inhibition of ectopic calcification *in vivo* by CAII expressing foreign body giant cells (FBGC) and macrophages

This example demonstrates that osteopontin promotes the accumulation and activation of carbonic anhydrase II expressing macrophages and osteoclast-like foreign body giant cells (FBGC) capable of acidifying the extracellular milieu and dissolving mineralized deposits.

Implantation of Aortic Valve Leaflets into OPN-Deficient Mice

The effect of subcutaneous implantation of a bioprosthetic glutaraldehyde-fixed aortic valve (GFAV) in normal mice and mice deficient in osteopontin was tested to further characterize the role of osteopontin in ectopic calcification *in vivo*. As described in Example V, mice deficient in one or both copies of the osteopontin gene were obtained according to Liaw et al., supra (1998). A 4.0 mm^2 piece of glutaraldehyde-fixed aortic valve leaflet (GFAV) was subcutaneously implanted into 5-6 week old female mice carrying either the wild type (WT), heterozygote (HTZ) or null allele (KO) for osteopontin. After 7, 14, and 30 days, implants were removed from osteopontin wild type (WT), heterozygote (HTZ) or null allele (KO) mice and assayed for mineral deposition, protein accumulation, and cell recruitment.

Once removed, the implants were fixed with methyl carnoys solution, embedded in paraffin, and 5μm sections were analyzed for OPN accumulation using an anti-OPN (OP-199) antibody at 10 μg/ml as described by Liaw et al., *supra*. Sections were counter stained with methyl green. OPN accumulation was quantitated using the Pro Image Analysis Program. In addition, calcium levels were assayed as described in Example I and normalized to the dried weight of the implant.

As shown in Figure 10A, osteopontin is observed within valves after 14 days of implantation into osteopontin WT mice. At this time, osteopontin is restricted to the border between the implant and the surrounding foreign body response. In contrast, the

osteopontin HTZ and KO mice have greatly reduced or absent OPN levels at 14 days of GFAV implantation, respectively.

To quantitate GFAV calcification, the explants were freeze dried, weighed, then acid hydrolyzed with 0.6 N HCL overnight at room temperature. Calcium quantitation was performed by the o-cresolphtalein complexone as directed in the Sigma Diagnostic kit (Sigma, St. Louis, IL) and normalized to dry weight. Accuracy of the kit was confirmed by atomic absorption spectroscopy. The osteopontin WT mice show no detectable GFAV mineralization based on Alizarin Red S staining and calcium quantitation. In contrast, the osteopontin HTZ and KO mice show GFAV calcification 4-to 5-fold higher than the osteopontin WT mice (Figure 10B).

Within 30 days, the osteopontin KO mice still do not accumulate osteopontin and GFAV mineralization is not significantly changed from the 14 day time point (Figures 10A and 10B). In the osteopontin HTZ and WT mice show elevated osteopontin levels with respect to the 14 day time point. In these mice, osteopontin localization is no longer restricted to the border of the implant/foreign body capsule, but is also found to penetrate the GFAV implant. In addition, the osteopontin localized to the foreign body capsule is most highly expressed by the FBGCs adjacent to the implant. At the 30 day time point, osteopontin WT mice exhibit GFAV calcification, but mineral levels are 4-fold lower than observed for osteopontin HTZ and KO mice. Also at the 30 day time point, osteopontin HTZ mice demonstrate a dramatic reduction in calcification, demonstrating that the presence of osteopontin is not only inhibiting mineral deposition, but also mediating the removal of

mineral from the GFAV. At the 60 day time point, osteopontin HTZ and WT mice exhibit a 4-5 fold increase compared to the respective 30 day time point (Figure 10B). Similarly, at the 60 day timepoint, GFAV valve calcification in osteopontin KO mice increases almost 2-fold compared to the 30 day time point (Figure 10B).

The accumulation of significant osteopontin levels observed at the 30 day timepoint in osteopontin HTZ mice was concurrent with a reduction or reversal in GFAV mineralization. Since OPN alone cannot mediate the dissolution of calcium phosphate, the observed mineral loss is rather an osteopontin-regulated event. The only mechanisms capable of calcium phosphate removal are phagocytosis and acidification.

Analysis of Osteopontin-Mediated Macrophage Recruitment

To examine macrophage accumulation GFAV implants removed from osteopontin WT, HTZ and KO mice at 14 days and 30 days and 5 µm sections were analyzed for macrophage accumulation using rat anti-mouse BM-8 (Accurate Chemical & Scientific Corp., Westbury, N.Y.) at 6 µg/ml. Following a 1 hour incubation with primary antibody, biotinylated goat anti-rat antibody (Vector Laboratories Inc., Burlingame, CA) was added and after 45 minutes the reaction product was detected with 3,3'-diaminobenzidine (DAB)(Sigma, St. Louis, MO). The sections were counterstained with methyl green

As represented by the immunochemical localization and quantitation of the BM-8 surface marker, the osteopontin KO mice display a defect in macrophage recruitment to the GFAV implantation site (Figure 11).

In addition, the osteopontin KO mice demonstrated BM-8 cell staining at levels 50%-25% lower compared to the osteopontin WT mice at 14 and 30 days of implantation, respectively.

In osteopontin WT mice, BM-8 positive cells accumulated diffusively at early time points, but later became concentrated along the edge of the implant suggesting their activation. In contrast, the BM-8 cells failed to become concentrated along the implant/foreign body capsule border in the osteopontin KO mice implying an activation defect.

While osteopontin HTZ mice also displayed decreased BM-8 positive cell accumulation, the macrophage defect did not correlate with the mineral loss pattern observed at the 30 day time point. Overall, very few macrophages were able to penetrate the GFAV at any time point examined, suggesting that phagocytosis alone cannot mediate mineralization reversal.

GFAV Explant pH Analysis

GFAV were explanted from osteopontin WT, HTZ, and KO mice after 30 days of implantation to investigate whether osteopontin promotes implant acidification, contributing to the removal of mineral from the GFAV implant as observed in osteopontin HTZ mice. The explants were freeze-dried and then immersed in Universal Indicator Solution (Fisher Scientific, Pittsburgh, PA). The pH of each implant was independently determined by three different observers comparing the color of the GFAV solution to the manufacturer's provided color scale.

While GFAV explants from the osteopontin WT mice had an acidic pH (6.0), explants from osteopontin KO mice maintained a near neutral pH (6.7). These differences in pH were statistically significant (p=.0002). GFAV implants from osteopontin HTZ mice had an intermediate pH (6.2). Unimplanted GFAV had a pH of 6.5 (Figure 12). Since apatite stability decreases at pHs less than 7.0 at 37° C, the observed pHs of the explants would be physiologically relevant with the respect to the ability to dissolve apatitic mineral deposits and explain the observed loss in GFAV mineralization. These findings demonstrate that osteopontin controls mineral resorption by regulating the ability of host cells to acidify the GFAV microenvironment.

Analysis of Carbonic Anhydrase II Expression at the GFAV Implantation Site

Osteoclasts and macrophages express carbonic anhydrase II (CAII), an enzyme that promotes acidification of the local microenvironment. CAII and cathepsin K expressing cells were localized in GFAV explants from osteopontin WT, HTZ, and KO mice at 14 days and 30 days after implantation by localizing protein in 5 µm sections using a sheep anti-human CAII antibody (Biodesign International, Kennebunkport, ME) at 5µg/ml. Incubation with primary antibody at room temperature for 1 hour was followed by incubation with a biotinylated rabbit anti-sheep antibody (Vector Laboratories Inc., Burlingame, CA) for 45 minutes. The reaction product was detected with 3,3'-diaminobenzidine (DAB)(Sigma, St. Louis, MO) and sections were counterstained with methyl green. CAII expression was quantitated using the Pro Image Analysis Program and is presented as percent area

in Figure 13. FBGC were counted in 4 quadrants of the explant (Figure 14). Any cell containing more than one nucleus was considered a FBGC.

Quantitation of CAII immunostaining revealed a mineral- and osteopontin dependent regulation of CAII expression (Figure 13). At 14 days, osteopontin is present and GFAV implants do not mineralize in osteopontin WT mice. Subsequently, when there is little requirement for mineral removal, CAII levels are low. At 30 days GFAV begin to mineralize, osteopontin accumulates, and CAII expressing cells are dramatically increased. Conversely, the osteopontin KO mice, which display elevated mineral levels at all observed time points, show very low CAII levels. The osteopontin mediated CAII response is accelerated in the osteopontin HTZ mice. At 14 days, osteopontin HTZ mice demonstrate substantial GFAV mineralization due to depressed levels of osteopontin, but respond to the mineralization by producing CAII-positive cells. Thus, the data demonstrate that osteopontin HTZ mice do not express high enough levels osteopontin to directly inhibit mineralization, but the expression level is sufficient to promote CAII expression or promotion of CAII-positive cell formation. In addition to CAII, high expression of cathepsin K, a cysteine protease expressed predominantly in osteoclasts and shown to be critical for osteoclast resorption activity, was observed in CAII-expressing FBGC.

To determine whether the acidification deficiency observed in osteopontin HTZ and osteopontin KO mice was a consequence of a reduced number of FBGCs, the number of FBGCs on GFAV explants was counted. FBGCs were visualized by electron microscopy performed as described

by Wada et al., Cir. Res. 84, 166-178 (1999), which is incorporated herein by reference. The number of FBGCs is enhanced 2-3 fold in OPN-deficient, highly calcified conditions represented by 14 day GFAV explants from osteopontin HTZ and KO mice.

The pattern of FBGC accumulation is almost identical to the calcium deposition pattern, and inverse to OPN accumulation. This data suggests that osteopontin deficiency promotes an increase in FBGC accumulation near the GFAV implant, but these FBGC demonstrate reduced acidicfication potential as measured by decreased CAII levels.

These results indicate that osteopontin promotes the accumulation and activation of carbonic anhydrase II expressing macrophages and osteoclast-like foreign body giant cells (FBGC) capable of acidifying the extracellular milieu and dissolving mineralized deposits.

Phosphorylated Osteopontin Inhibits Bioprosthetic Valve Calcification *In Vivo*

GFAV were incubated in a 1 mg/ml solution of either control vehicle, unphosphorylated osteopontin or phosphorylated osteopontin for three days prior to implantation into osteopontin KO mice. After 14 days calcium quantitation was performed as described above. As shown in Figure 15, these results indicate that phosphorylated osteopontin inhibits bioprosthetic valve calcification *in vivo*.

What is claimed is:

1. A method of inhibiting ectopic calcification in an individual, comprising administering to said individual a therapeutically effective amount of osteopontin or a functional fragment thereof.

2. The method of claim 1, wherein said ectopic calcification is associated with a condition selected from the group consisting of atherosclerosis, stenosis, restenosis, prosthetic valve replacement, angioplasty, renal failure, tissue injury, diabetes and aging.

3. The method of claim 1, wherein said osteopontin is a polypeptide comprising substantially the amino acid sequence of SEQ ID NO:2, or a functional fragment thereof.

4. The method of claim 1, wherein said osteopontin or functional fragment thereof is administered with a pharmaceutically acceptable carrier.

5. The method of claim 1, wherein said osteopontin or functional fragment thereof is administered at the site of ectopic calcification.

6. The method of claim 5, wherein said osteopontin or functional fragment thereof contacts a prosthetic device.

7. The method of claim 6, wherein said prosthetic device is a bioprosthetic heart valve.

8. The method of claim 6, wherein said contacting comprises attachment of osteopontin or a functional fragment thereof to said prosthetic device.

9. The method of claim 6, wherein said contacting comprises attachment of cells producing said osteopontin or a functional fragment thereof to said prosthetic device.

10. The method of claim 9, wherein said cells recombinantly produce osteopontin or a functional fragment thereof.

11. The method of claim 1, wherein said osteopontin interacts with hydroxyapatite crystals.

12. The method of claim 1, wherein said osteopontin stimulates cells to acidify the local microenvironment.

13. The method of claim 12, wherein said cells express carbonic anhydrase II.

14. The method of claim 13, wherein said cells are multinucleate foreign body giant cells.

15. The method of claim 13, wherein said cells are macrophages.

16. A method of treating or inhibiting ectopic calcification comprising administering to an individual macrophages, whereby said macrophages are targeted to the site of ectopic calcification.

17. The method of claim 16, wherein said macrophages acidify the local microenvironment by expressing carbonic anhydrase II.

18. The method of claim 16, further comprising administering osteopontin, thereby stimulating said acidification of the local microenvironment by said macrophages.

19. The method of claim 16, wherein said macrophages contact a prosthetic device.

20. The method of claim 19, wherein said prosthetic device is a bioprosthetic heart valve.

21. A method of treating or inhibiting ectopic calcification comprising administering to an individual multinucleate foreign body giant cells, whereby said multinucleate foreign body giant cells are targeted to the site of ectopic calcification.

22. The method of claim 21, wherein said multinucleate foreign body giant cells acidify the local microenvironment by expressing carbonic anhydrase II.

23. The method of claim 21, further comprising administering osteopontin, thereby stimulating said acidification of the local microenvironment by said multinucleate foreign body giant cells.

24. The method of claim 21, wherein said multinucleate foreign body giant cells contact a prosthetic device.

25. The method of claim 24, wherein said prosthetic device is a bioprosthetic heart valve.

26. A method of treating or inhibiting ectopic calcification in an individual comprising promoting recruitement of acid producing cells to a site of ectopic calcification by administering osteopontin.

27. The method of claim 26, wherein said acid producing cells express carbonic anhydrase II.

28. The method of claim 26, wherein said acid producing cells are macrophages.

29. The method of claim 26, wherein said acid producing cells are multinucleate foreign body giant cells.

30. A method of treating or inhibiting ectopic calcification in an individual comprising increasing expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin.

31. The method of claim 30, wherein carbonic anhydrase II is expressed by multinucleate foreign body giant cells.

32. The method of claim 30, wherein carbonic anhydrase II is expressed by macrophages.

a)

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   1  GACCAGACTC GTCTCAGGCC AGTTGCAGCC TTCTCAGCCA AACGCCGACC AAGGAAAACT CACTACCATG AGAATTGCAG TGATTTGCTT TTGCCTCCTA
 101  GGCATCACCT GTGCCATACC AGTTAAACAG GCTGATTCTG GAAGTTCTGA GGAAAAGCAG CTTTACAACA AATACCCAGA TGCTGTGGCC ACATGGCTAA
 201  ACCCTGACCC ATCTCAGAAG CAGAATCTCC TAGCCCCACA GAATGCTGTG TCCTCTGAAG AAACCAATGA CTTTAAACAA GAGACCCTTC CAAGTAAGTC
 301  CAACGAAAGC CATGACCACA TGGATGATAT GGATGATGAA GATGATGATG ACCATGTGGA CAGCCAGGAC TCCATTGACT CGAACGACTC TGATGATGTA
 401  GATGACACTG ATGATTCTCA CCAGTCTGAT GAGTCTCACC ATTCTGATGA ATCTGATGAA CTGGTCACTG ATTTTCCCAC GGACCTGCCA GCAACCGAAG
 501  TTTTCACTCC AGTTGTCCCC ACAGTAGACA CATATGATGG CCGAGGTGAT AGTGTGGTTT ATGGACTGAG GTCAAAATCT AAGAAGTTTC GCAGACCTGA
 601  CATCCAGTAC CCTGATGCTA CAGACGAGGA CATCACCTCA CACATGGAAA GCGAGGAGTT GAATGGTGCA TACAAGCCCA TCCCCGTTGC CCAGGACCTG
 701  AACGCGCCTT CTGATTGGGA CAGCCGTGGG AAGGACAGTT ATGAAACGAG TCAGCTGGAT GACCAGAGTG CTGAAACCCA CAGCCACAAG CAGTCCAGAT
 801  TATATAAGCG GAAAGCCAAT GATGAGAGCA ATGAGCATTC CGATGTGATT GATAGTCAGG AACTTTCCAA AGTCAGCCGT GAATTCCACA GCCATGAATT
 901  TCACAGCCAT GAAGATATGC TGGTTGTAGA CCCCAAAAGT AAGGAAGAAG ATAAACACCT GAAATTTCGT ATTTCTCATG AATTAGATAG TGCATCTTCT
1001  GAGGTCAATT AAAAGGAGAA AAAATACAAT TTCTCACTTT GCATTTAGTC AAAAGAAAAA ATGCTTTATA GCAAAATGAA AGAGAACATG AAATGCTTCT
1101  TTCTCAGTTT ATTGGTTGAA TGTGTATCTA TTTGAGTCTG GAAATAACTA ATGTGTTTGA TAATTAGTTT AGTTTGTGGC TTCATGGAAA CTCCCTGTAA
1201  ACTAAAAGCT TCAGGGTTAT GTCTATGTTC ATTCTATAGA AGAAATGCAA ACTATCACTG TATTTTAATA TTTGTTATTC TCTCATGAAT AGAAATTTAT
1301  GTAGAAGCAA ACAAAATACT TTTACCCACT TAAAAAGAGA ATATAACATT TTATGTCACT ATAATCTTTT GTTTTTTAAG TTAGTGTATA TTTTGTTGTG
1401  ATTATCTTTT TGTGGTGTGA ATAA
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b)

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Human 1  MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDD-DHVDS

   101  QDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITSHMESEELN

   201  GAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKANDESNEHSDV----------IDSQELSKVSREFHSHEFHSH

   301  EDMLVVDPKSKEEDKHLKFRISHELDSASSEVN
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FIGURE 1

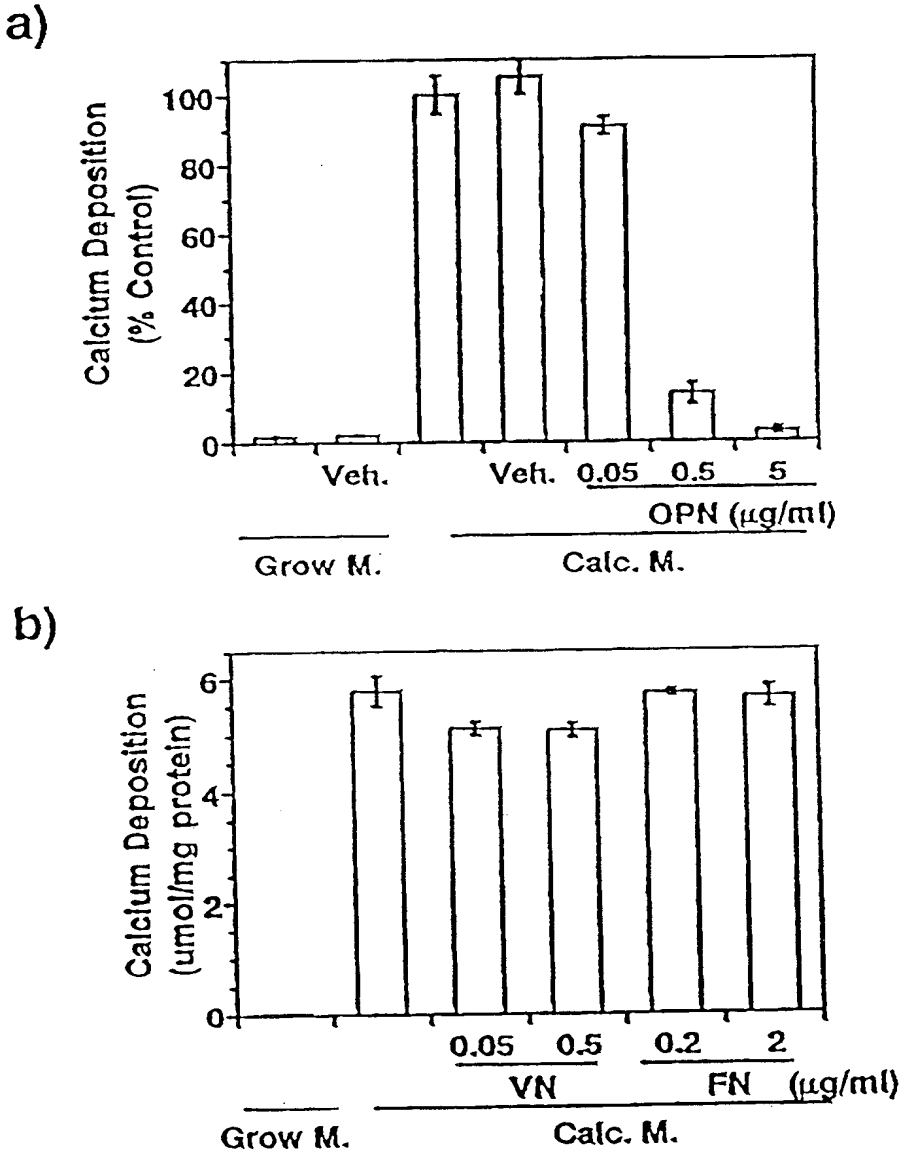

a)
Calcium Deposition
(% Control)
100
80
60
40
20
0
Veh.
Veh.
0.05
0.5
5
OPN (μg/ml)
Grow M.
Calc. M.
b)
Calcium Deposition
(umol/mg protein)
6
4
2
0
0.05
0.5
0.2
2
VN
FN
(μg/ml)
Grow M.
Calc. M.

FIGURE 2

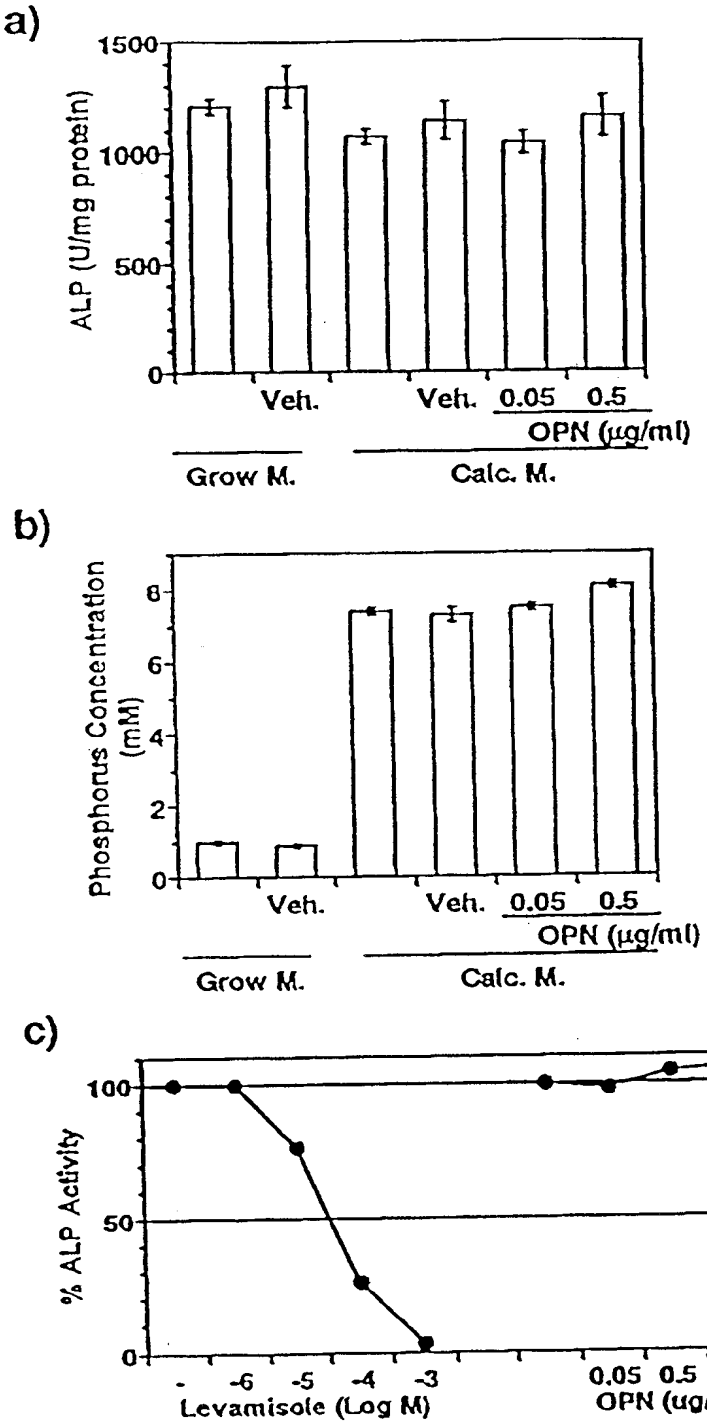

a)
ALP (U/mg protein)
1500
1000
500
0
Veh.
Veh.
0.05
0.5
OPN (µg/ml)
Grow M.
Calc. M.
b)
Phosphorus Concentration (mM)
8
6
4
2
0
Veh.
Veh.
0.05
0.5
OPN (µg/ml)
Grow M.
Calc. M.
c)
% ALP Activity
100
50
0
-
-6
-5
-4
-3
Levamisole (Log M)
0.05
0.5
5
OPN (ug/ml)

FIGURE 3

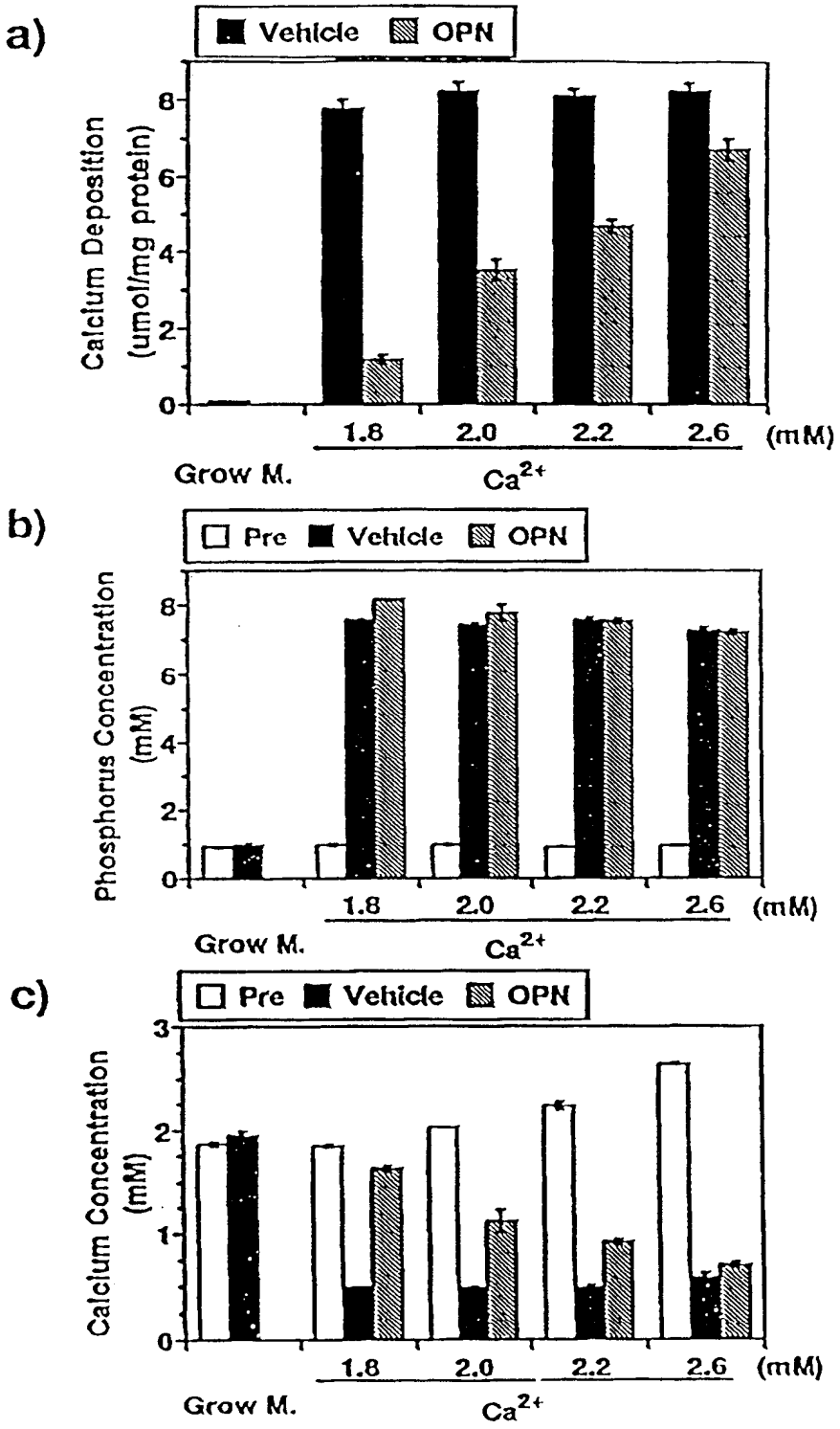
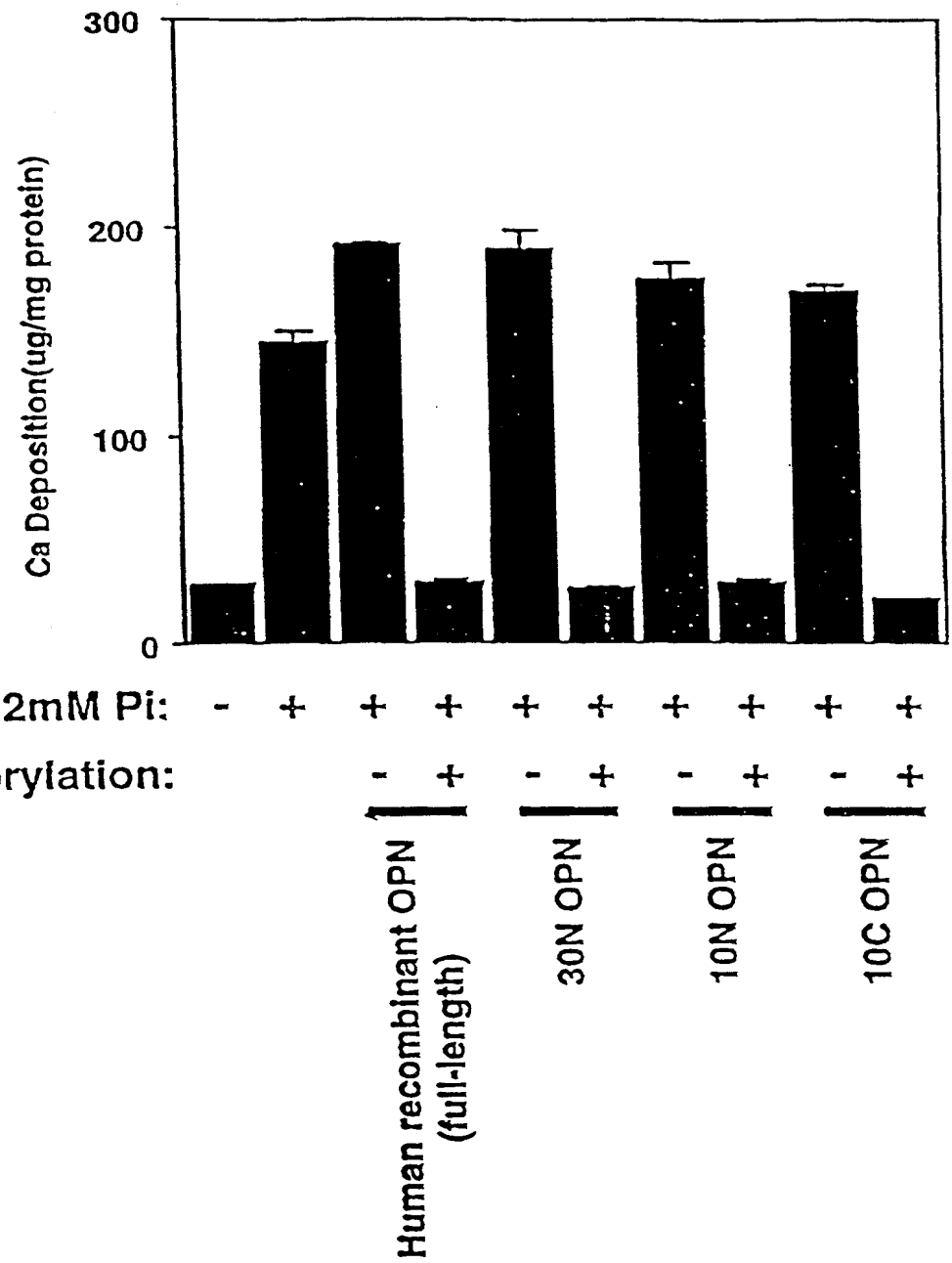

a)
Vehicle
OPN
Calcium Deposition (umol/mg protein)
0
2
4
6
8
1.8
2.0
2.2
2.6
(mM)
Grow M.
Ca2+
b)
Pre
Vehicle
OPN
Phosphorus Concentration (mM)
0
2
4
6
8
1.8
2.0
2.2
2.6
(mM)
Grow M.
Ca2+
c)
Pre
Vehicle
OPN
Calcium Concentration (mM)
0
1
2
3
1.8
2.0
2.2
2.6
(mM)
Grow M.
Ca2+

FIGURE 4

a)



b)

OPN	mol of phosphate/mol of OPN
Full-length	20
30N	12
10N	8.9
10C	8.9

FIGURE 5

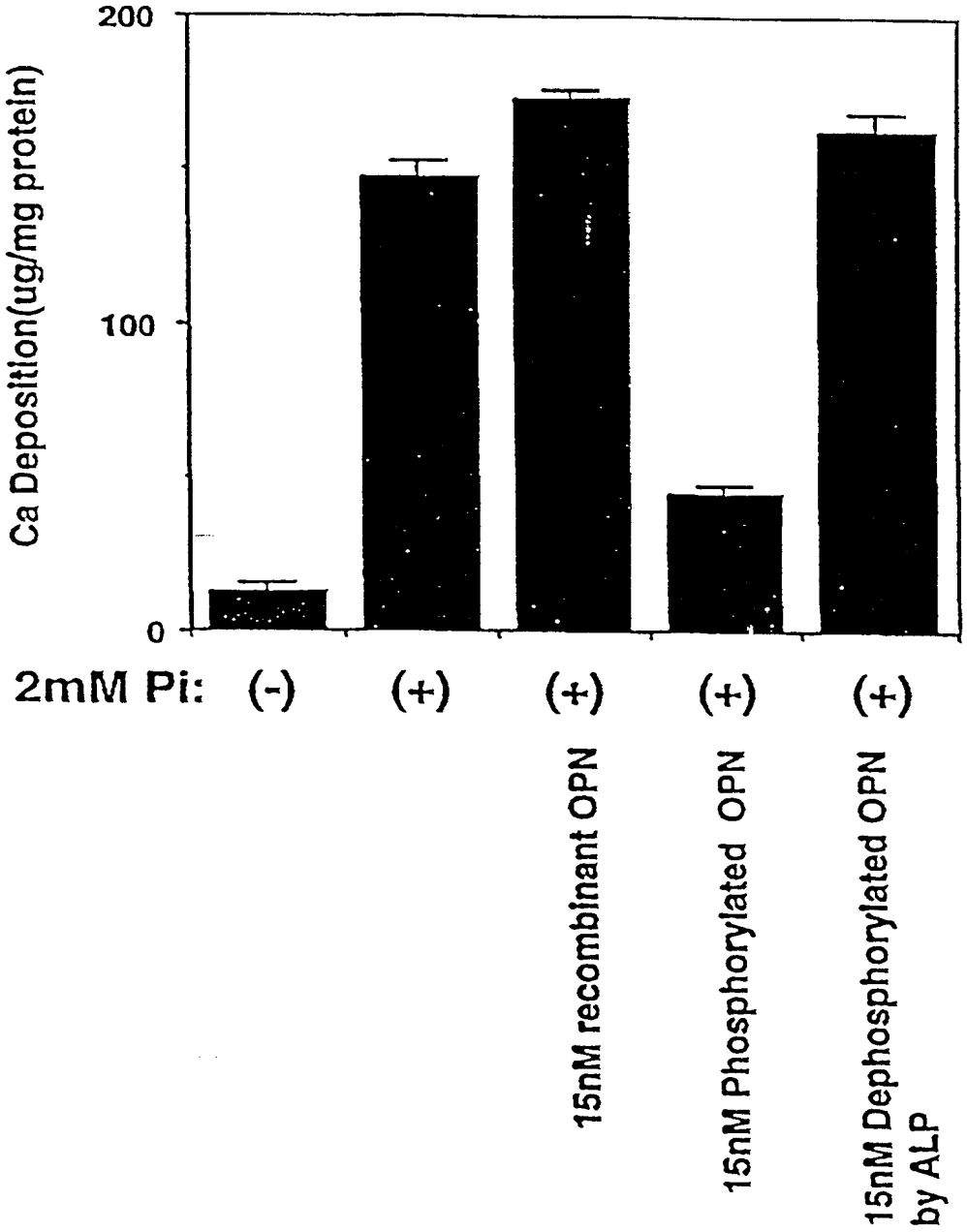

200
100
0
Ca Deposition(ug/mg protein)
2mM Pi: (-) (+) (+) (+) (+)
15nM recombinant OPN
15nM Phosphorylated OPN
15nM Dephosphorylated OPN by ALP

FIGURE 6

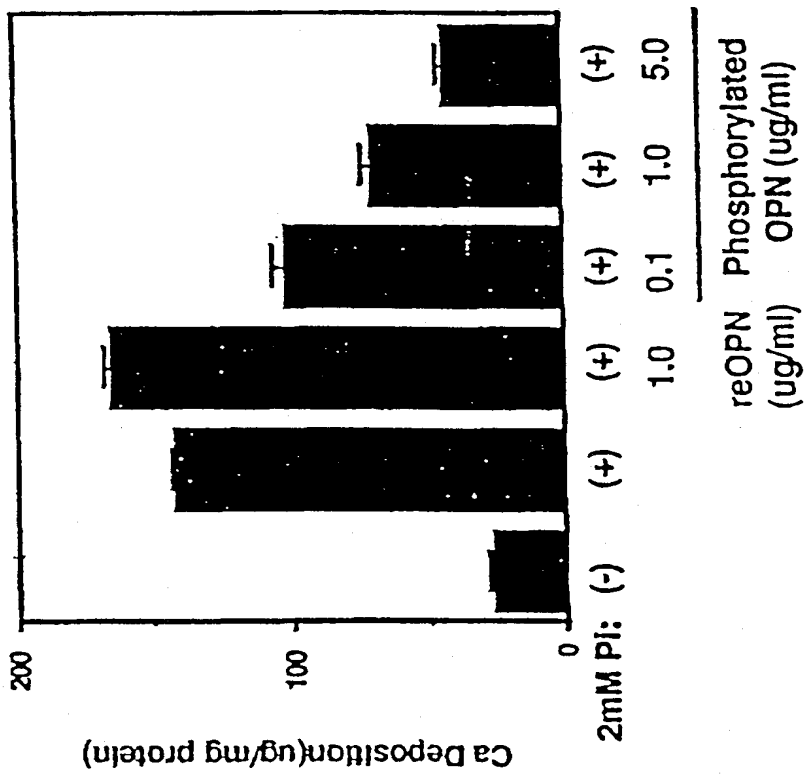

Ca Deposition(ug/mg protein)
200
100
0
2mM Pi: (-) (+) (+) (+) (+) (+)
reOPN (ug/ml) 1.0 0.1 1.0 5.0
Phosphorylated OPN (ug/ml)

FIGURE 7

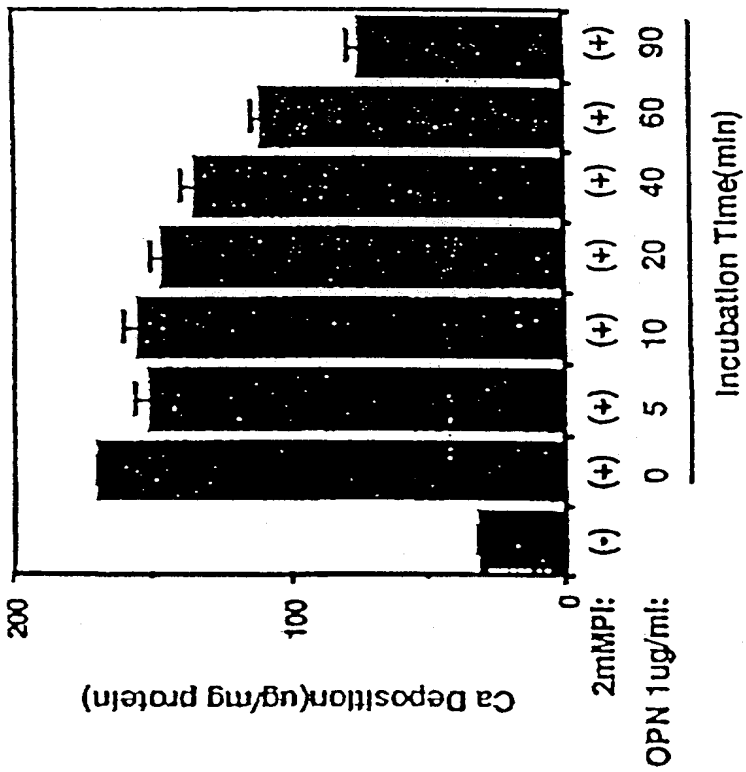

Ca Deposition(ug/mg protein)
200
100
0
2mMPI: (-) (+) (+) (+) (+) (+) (+) (+)
OPN 1ug/ml:
0 5 10 20 40 60 90
Incubation Time(min)

FIGURE 8

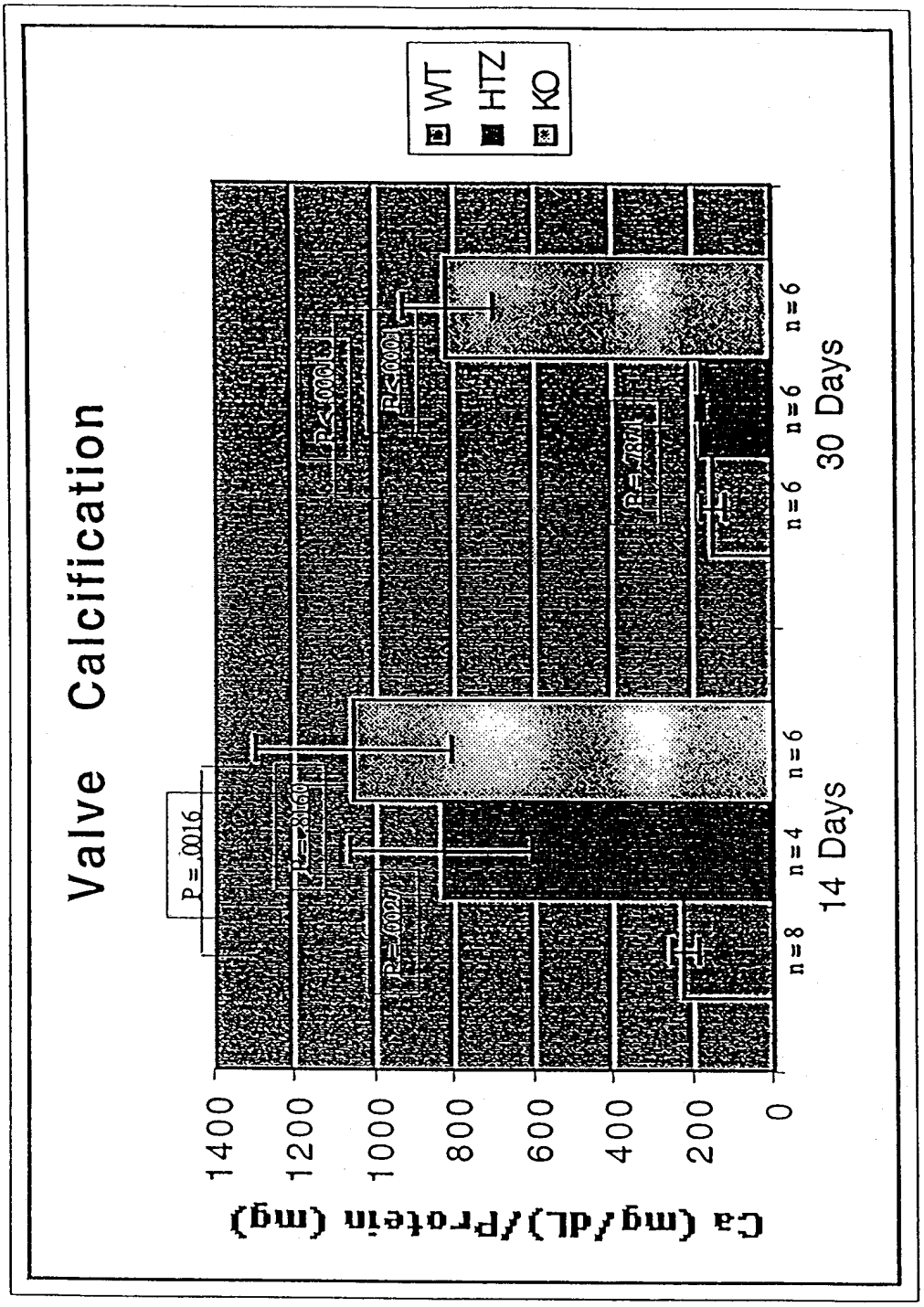

Valve Calcification
WT
HTZ
KO
Ca (mg/dL)/Protein (mg)
1400
1200
1000
800
600
400
200
0
P = .0016
n=8
n=4
n=6
n=6
n=6
n=6
14 Days
30 Days

FIGURE 9

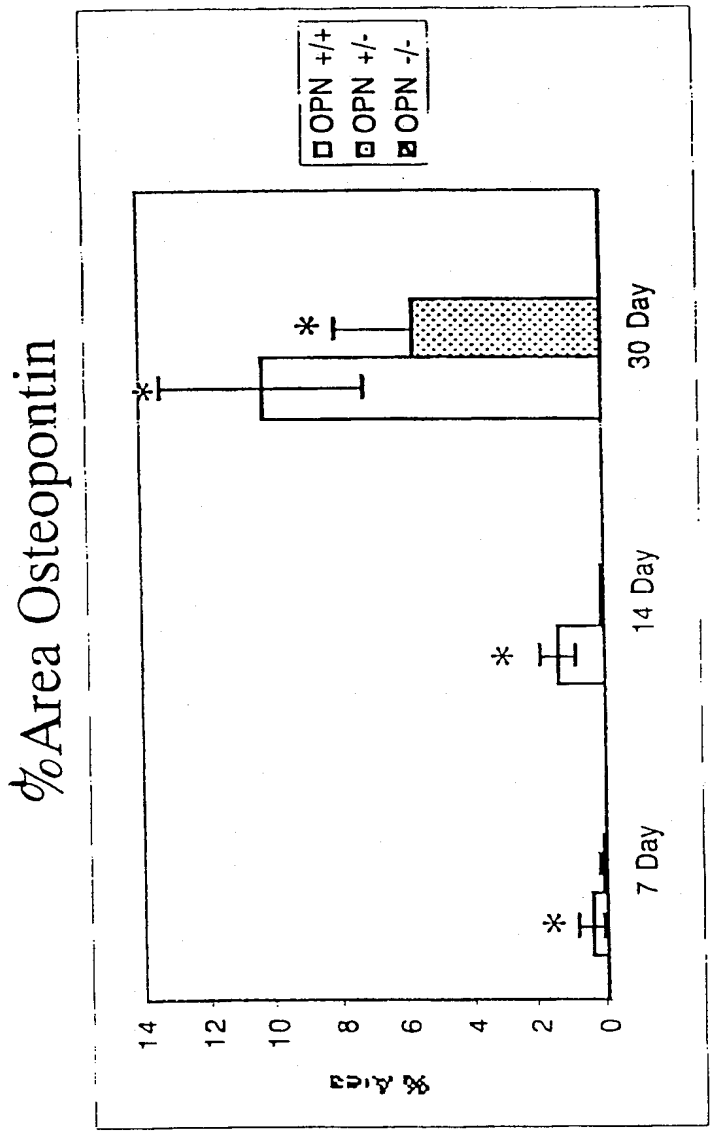


FIGURE 10A

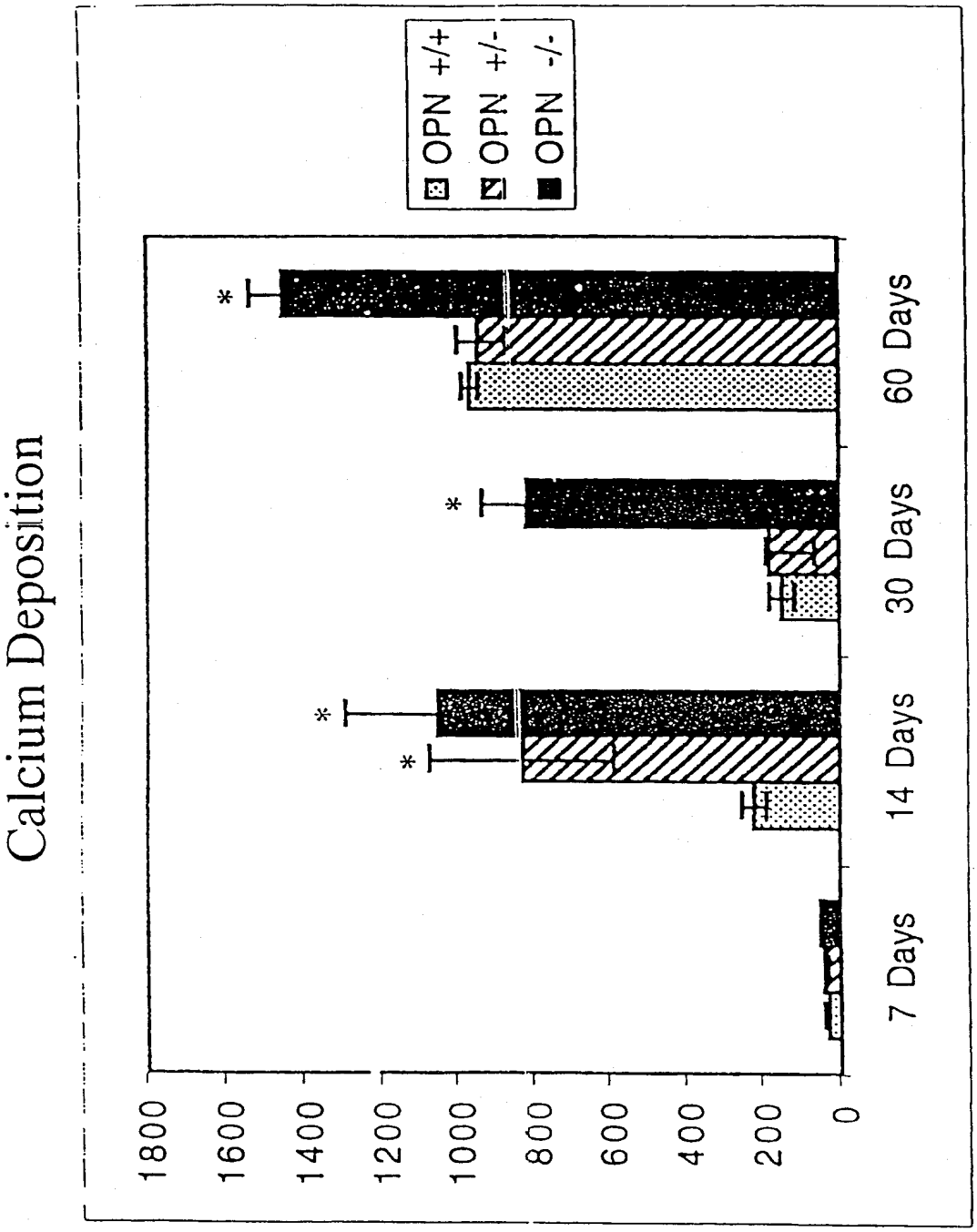

Calcium Deposition
OPN +/+
OPN +/-
OPN -/-
1800
1600
1400
1200
1000
800
600
400
200
0
7 Days
14 Days
30 Days
60 Days
*
*
*
*

FIGURE 10B

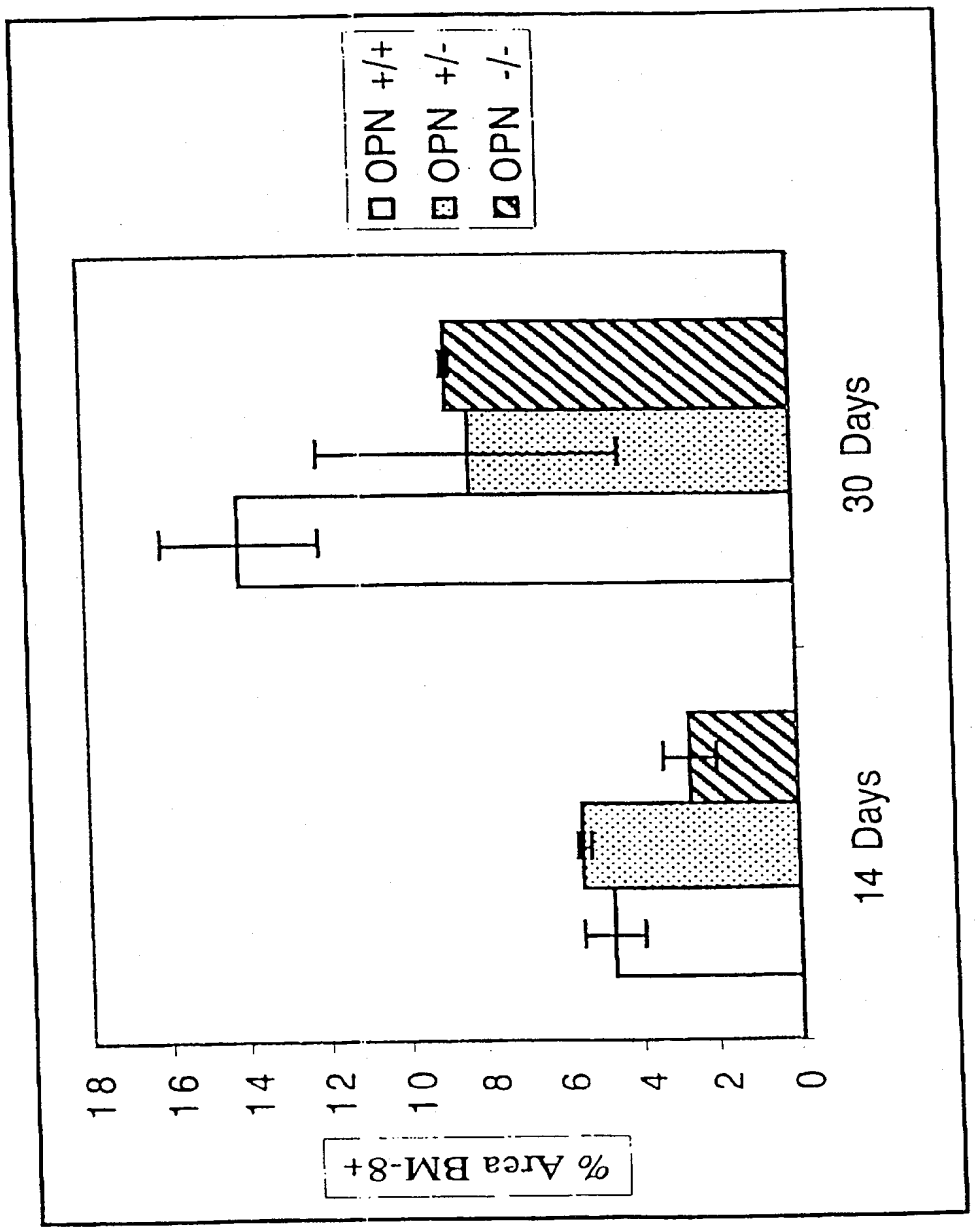


FIGURE 11

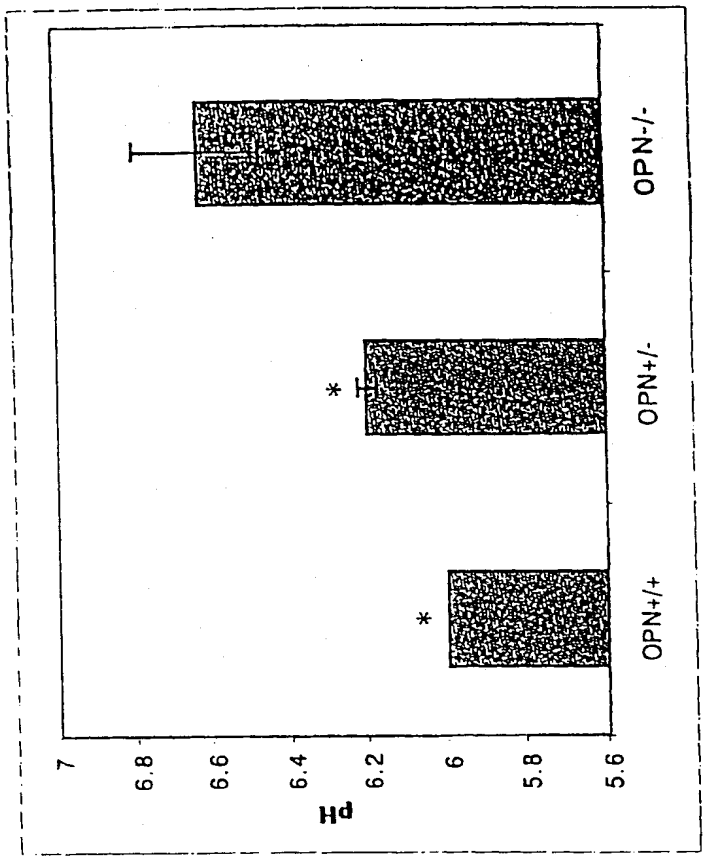


FIGURE 12

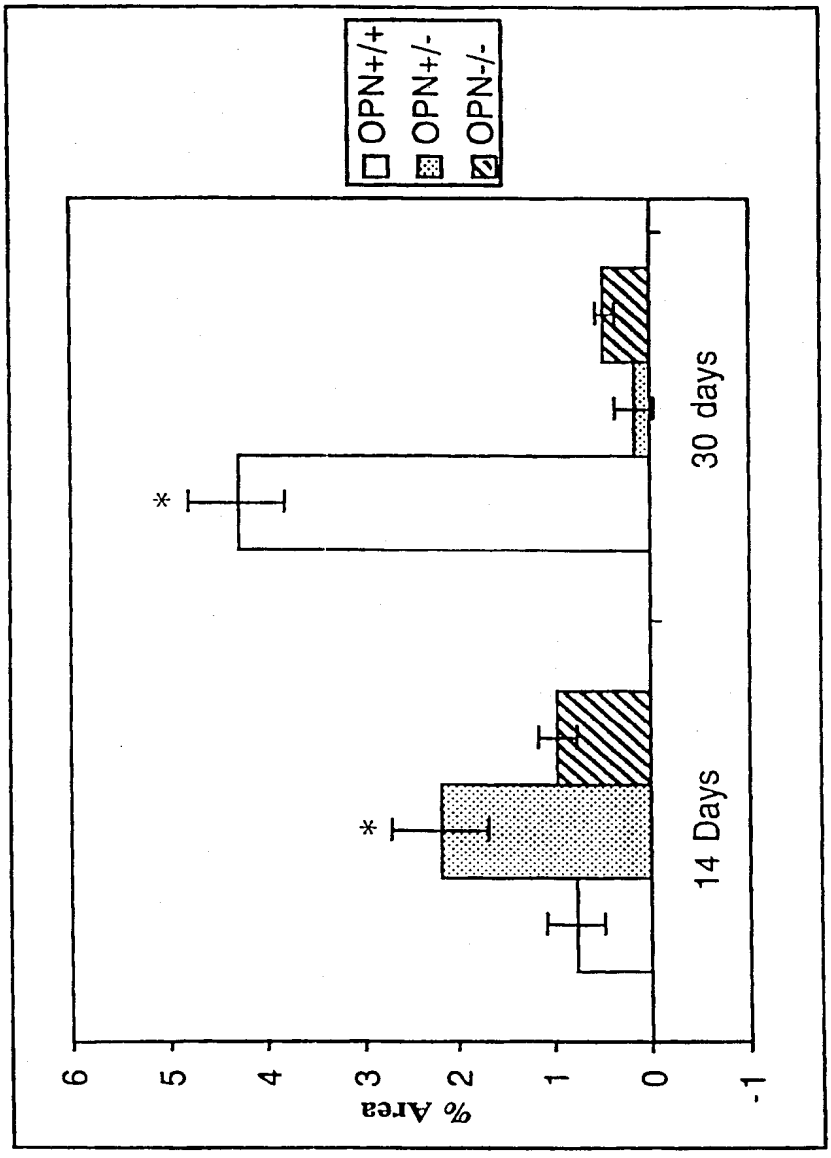


FIGURE 13

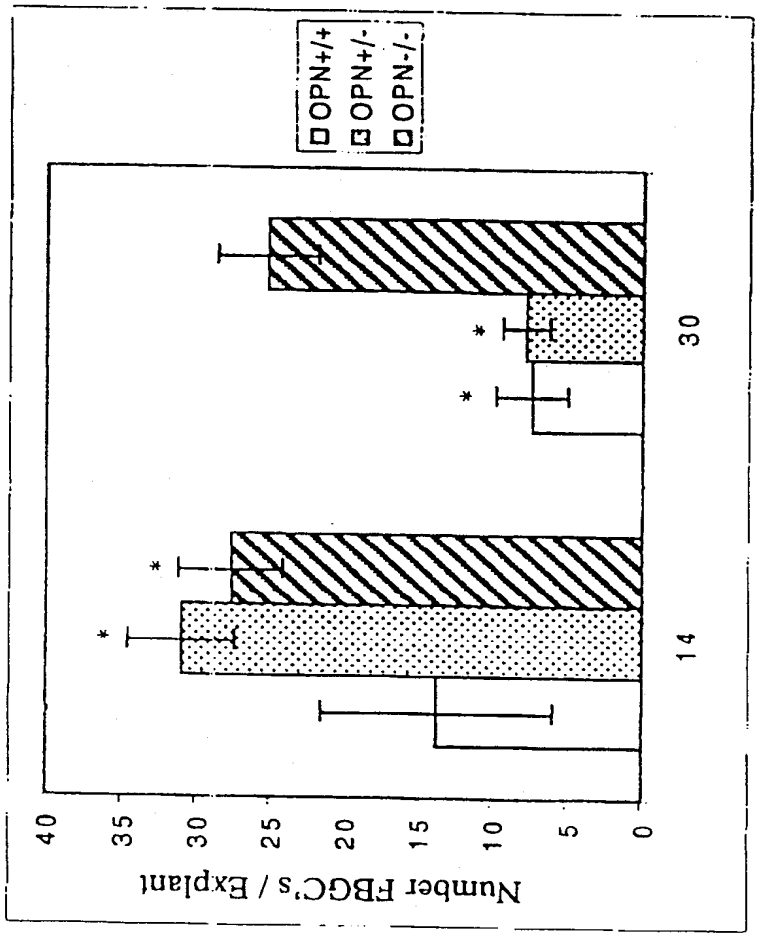


FIGURE 14

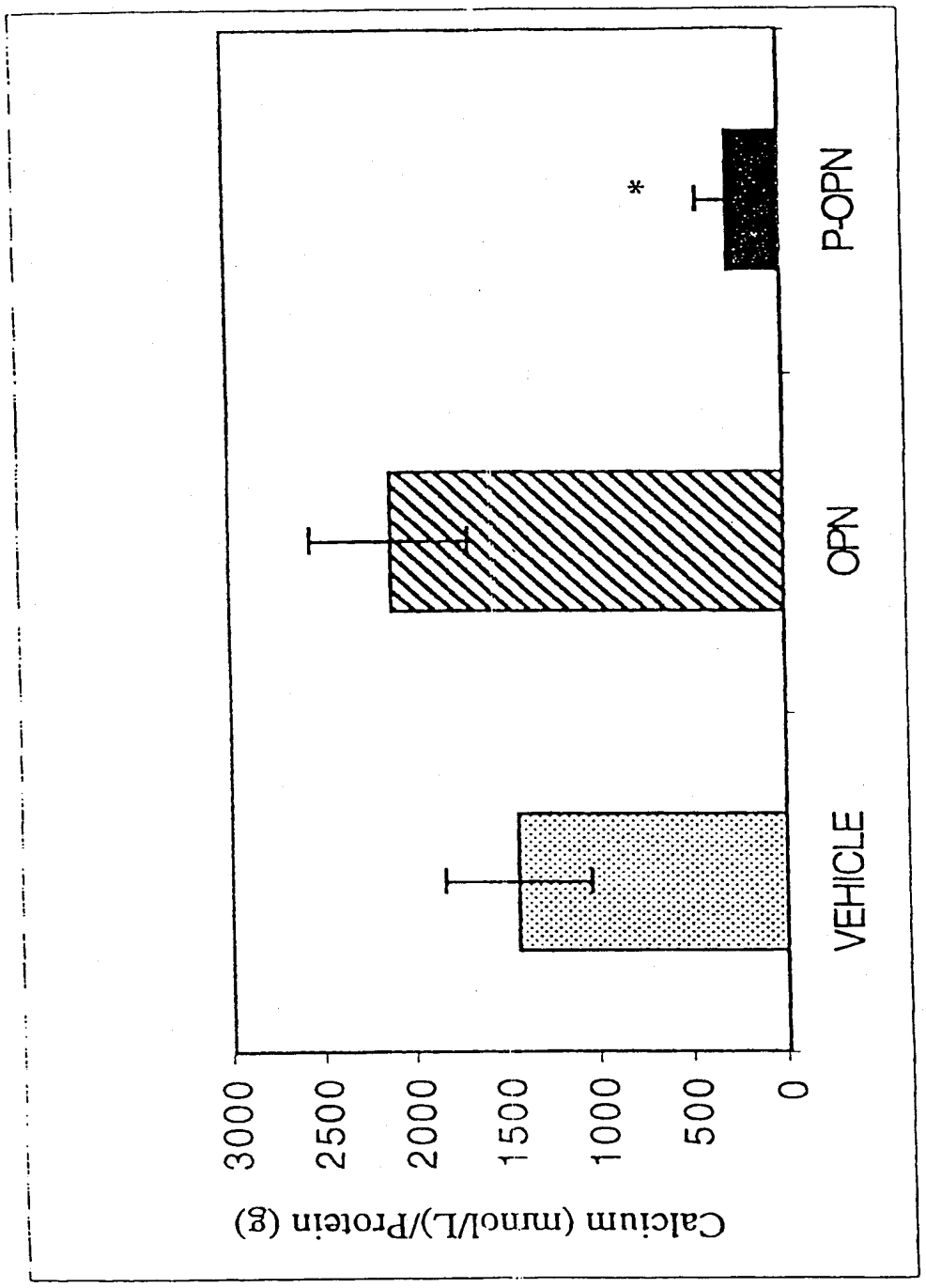

Calcium (mmol/L)/Protein (g)
3000
2500
2000
1500
1000
500
0
VEHICLE
OPN
P-OPN
*

FIGURE 15

SEQUENCE LISTING

<110> Giachelli, Cecilia M.
Steitz, Susie
University of Washington

<120> Methods of Inhibiting Ectopic Calcification

<130> FP-UW 3801

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<150> US 09/206,576
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Gln Leu Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro
35 40 45

gac cca tct cag aag cag aat ctc cta gcc cca cag aat gct gtg tcc 253
Asp Pro Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser
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Ser Glu Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser
         65                  70                  75

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Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp
     80                  85                  90

gac cat gtg gac agc cag gac tcc att gac tcg aac gac tct gat gat   397
Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp
 95                 100                 105                 110

gta gat gac act gat gat tct cac cag tct gat gag tct cac cat tct   445
Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser
                115                 120                 125

gat gaa tct gat gaa ctg gtc act gat ttt ccc acg gac ctg cca gca   493
Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala
            130                 135                 140

acc gaa gtt ttc act cca gtt gtc ccc aca gta gac aca tat gat ggc   541
Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly
        145                 150                 155

cga ggt gat agt gtg gtt tat gga ctg agg tca aaa tct aag aag ttt   589
Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe
    160                 165                 170

cgc aga cct gac atc cag tac cct gat gct aca gac gag gac atc acc   637
Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr
175                 180                 185                 190

tca cac atg gaa agc gag gag ttg aat ggt gca tac aag gcc atc ccc   685
Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro
                195                 200                 205

gtt gcc cag gac ctg aac gcg cct tct gat tgg gac agc cgt ggg aag   733
Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys
            210                 215                 220

gac agt tat gaa acg agt cag ctg gat gac cag agt gct gaa acc cac   781
Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His
        225                 230                 235

agc cac aag cag tcc aga tta tat aag cgg aaa gcc aat gat gag agc   829
Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser
    240                 245                 250
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aat gag cat tcc gat gtg att gat agt cag gaa ctt tcc aaa gtc agc   877
Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser
255                 260                 265                 270

cgt gaa ttc cac agc cat gaa ttt cac agc cat gaa gat atg ctg gtt   925
Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val
                275                 280                 285

gta gac ccc aaa agt aag gaa gaa gat aaa cac ctg aaa ttt cgt att   973
Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile
            290                 295                 300

tct cat gaa tta gat agt gca tct tct gag gtc aat taaaaggaga        1019
Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
        305                 310

aaaaatacaa tttctcactt tgcatttagt caaaagaaaa aatgctttat agcaaaatga 1079

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ttttacccac ttaaaaagag aatataacat tttatgtcac tataatcttt tgtttttaa 1379

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Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser Ser Glu
     50                  55                  60
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Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser Asn Glu
 65                  70                  75                  80

Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp Asp His
                 85                  90                  95

Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp
            100                 105                 110

Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser Asp Glu
        115                 120                 125

Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala Thr Glu
    130                 135                 140

Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly Arg Gly
145                 150                 155                 160

Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe Arg Arg
                165                 170                 175

Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His
            180                 185                 190

Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro Val Ala
        195                 200                 205

Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys Asp Ser
    210                 215                 220

Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His Ser His
225                 230                 235                 240

Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu
                245                 250                 255

His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser Arg Glu
            260                 265                 270

Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val Val Asp
        275                 280                 285

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INTERNATIONAL SEARCH REPORT

Inte. .donal Application No
PCT/US 99/29173

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/19 A61K35/14 A61P9/10 A61P13/12 A61P3/00
A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIACHELLI, CECILIA M. ET AL: "Osteopontin: Potential roles in vascular function and dystrophic calcification" J. BONE MINER. METAB. (1997), 15(4), 179-183 , XP000886502	1-15, 26-32
Y	page 181, column 2 ---	16-20
Y	O'BRIEN E R ET AL: "Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques." ARTERIOSCLEROSIS AND THROMBOSIS, (1994 OCT) 14 (10) 1648-56. , XP000886521 abstract discussion --- -/--	16-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 May 2000	24/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pilling, S

3

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INTERNATIONAL SEARCH REPORT

Inte. .donal Application No
PCT/US 99/29173

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	MURRY C E ET AL: "Macrophages express osteopontin during repair of myocardial necrosis." AMERICAN JOURNAL OF PATHOLOGY, (1994 DEC) 145 (6) 1450-62. , XP000886530 discussion abstract	16-20
Y	GIACHELLI, CECILIA M. ET AL: "Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo" AM. J. PATHOL. (1998), 152(2), 353-358 , XP000886540 discussion abstract	16-20
Y	DAOUD A S ET AL: "Sequential morphologic studies of regression of advanced atherosclerosis." ARCHIVES OF PATHOLOGY AND LABORATORY MEDICINE, (1981 MAY) 105 (5) 233-9. , XP000886554 abstract final paragraph of the comment on page 239	16-20
A	WO 92 22316 A (PHILADELPHIA CHILDREN HOSPITAL ;UNIV PENNSYLVANIA (US); UNIV CAMBR) 23 December 1992 (1992-12-23) page 9, line 21 -page 9, line 24 page 13, line 21 -page 13, line 23	1-32
A	US 5 695 761 A (HECK DIANE ELAINE ET AL) 9 December 1997 (1997-12-09) column 4, line 26 -column 4, line 27 column 4, line 43 -column 4, line 46	1-32
A	EP 0 705 842 A (HOECHST AG) 10 April 1996 (1996-04-10) abstract	1-32

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte .ional Application No
PCT/US 99/29173

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9222316 A	23-12-1992	US 5304496 A	19-04-1994
		AU 2186392 A	12-01-1993
US 5695761 A	09-12-1997	NONE	
EP 0705842 A	10-04-1996	AU 695117 B	06-08-1998
		AU 3308695 A	18-04-1996
		CA 2159957 A	07-04-1996
		JP 8191693 A	30-07-1996
		ZA 9508381 A	24-04-1996

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(19) World Intellectual Property Organization
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(10) International Publication Number
WO 00/033865 A1

(51) International Patent Classification[7]: A61K 38/19, 35/14, A61P 9/10, 13/12, 3/00, 43/00

(21) International Application Number: PCT/US99/29173

(22) International Filing Date: 7 December 1999 (07.12.1999)

(25) Filing Language: English

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(30) Priority Data:
09/206,576 7 December 1998 (07.12.1998) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/206,576 (CIP)
Filed on 7 December 1998 (07.12.1998)

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF WASHINGTON** [US/US]; Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GIACHELLI, Cecilia, M.** [US/US]; 3012 154th Street, S.E., Mill Creek, WA 98012 (US). **STEITZ, Susie** [US/US]; 95145 8th Avenue NW #206, Seattle, WA 98117 (US).

(74) Agents: **CADENA, Deborah, L.** et al.; Campbell & Flores LLP, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States *(national)*: AE, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR (utility model), KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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— *with international search report*

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WO 00/033865 A1

(54) Title: METHODS OF INHIBITING ECTOPIC CALCIFICATION

(57) Abstract: The invention provides a method of inhibiting ectopic calcification in an individual. The method consists of administratering to the invidual a therapeutically effective amount of osteopontin or a functional fragment thereof. The invention also provides a method of treating or inhibiting ectopic calcification by administering to an individual acid-producing cells targeted to the site of ectopic calcification. The invention further provides a method of treating or inhibiting ectopic calcification in an individual consisting of promoting recruitement of acid producing cells to a site of ectopic calcification by administering osteopontin. The invention also provides a method of treating or inhibiting ectopic calcification in an individual consisting of increasing expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin.

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METHODS OF INHIBITING ECTOPIC CALCIFICATION

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BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the field of medicine and, more specifically, to methods of inhibiting ectopic calcification.

BACKGROUND INFORMATION

Deposition of calcium crystals in tissues other than teeth or bone, referred to as ectopic calcification, commonly occurs in association with renal failure, cardiovascular disease, diabetes and the aging process. A frequent finding in patients with renal failure, particularly those undergoing long-term hemodialysis and unable to appropriately regulate serum mineral balance, is calcification of internal organs, including the lung, heart, stomach and kidneys. Less commonly, hemodialysis patients develop painful calcified skin lesions that progress to non-healing ulcers or gangrene and may require amputation of the affected limb.

Ectopic calcification is also a common complication of the implantation of bioprosthetic heart valves and is the leading cause of replacement valve

failure. Ectopic calcification also occurs in native heart valves and blood vessels in association with atherosclerosis, diabetes and cardiovascular disease. The deposition of minerals in the vasculature narrows the orifices and hardens the walls of the affected valves and blood vessels, resulting in reduced blood flow to the heart and peripheral organs. Therefore, ectopic calcification increases the risk of valve failure, stroke, ischemia and myocardial infarction.

One protein that is abundant at the sites of ectopic calcification, such as in atherosclerotic plaques and in calcified aortic valves, is osteopontin. Osteopontin has several known functions, including promoting cell adhesion, spreading and migration. Osteopontin colocalizes with sites of early calcification in coronary atherosclerotic plaques and its expression increases as atherosclerosis develops. These findings, combined with studies showing that osteopontin has calcium-binding properties *in vitro*, have led to the suggestion that osteopontin may be involved in ectopic calcification. Previous studies have not addressed the role of osteopontin in ectopic calcification *in vivo*.

Ectopic calcification, if left untreated, results in increased morbidity and death. Current therapies to normalize serum mineral levels or to inhibit calcification of vascular tissues or implants are of limited efficacy and cause unacceptable side effects.

Thus, there exists a need for an effective method of inhibiting ectopic calcification. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a method of inhibiting ectopic calcification in an individual. The method consists of administering to the individual a therapeutically effective amount of osteopontin or a functional fragment thereof. The method can be used to inhibit ectopic calcification associated with a variety of conditions such as atherosclerosis, stenosis, restenosis, prosthetic valve replacement, angioplasty, renal failure, tissue injury, diabetes and aging. The invention also provides a method of treating or inhibiting ectopic calcification by administering to an individual acid-producing cells targeted to the site of ectopic calcification. The invention further provides a method of treating or inhibiting ectopic calcification in an individual consisting of promoting recruitement of acid producing cells to a site of ectopic calcification by administering osteopontin. The invention also provides a method of treating or inhibiting ectopic calcification in an individual consisting of increasing expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of human osteopontin, as described by Kiefer et al., Nucleic Acids Res. 17:3306 (1989).

Figure 2 shows the effects of osteopontin (a) on calcification of BASMC as compared to vitronectin and fibronectin (b).

Figure 3 shows the effects of osteopontin on alkaline phosphatase activity of BASMC (a) and phosphorous concentration in the medium (b) and the effects of levamisole and osteopontin (OPN) on alkaline phosphatase (ALP) activity (c).

Figure 4 shows the effects of osteopontin on calcium deposition (a), medium phosphorous concentration (b) and medium calcium concentration (c) at various initial calcium concentrations.

Figure 5 shows the effects of recombinant osteopontin and its functional fragments on HSMC calcium deposition (a) and the extent of phosphorylation of recombinant osteopontin fragments by casein kinase II (b).

Figure 6 shows the effect of phosphorylation and dephosphorylation of osteopontin on HSMC calcification.

Figure 7 shows the effect of various concentrations of osteopontin on HSMC calcification.

Figure 8 shows the time course of osteopontin inhibition of HSMC calcification.

Figure 9 shows the effect of osteopontin gene copy number on calcification of valves implanted subcutaneously into mice.

Figure 10 shows the effect of osteopontin gene copy number on osteopontin accumulation on valves implanted subcutaneously into mice and explanted at 7, 14, and 30 day time points (a), and calcium deposition on

valves implanted subcutaneously into mice and explanted at 7, 14, 30, and 60 day time points (b).

Figure 11 shows the effect of osteopontin gene copy number on macrophage recruitment to the site of valves implanted subcutaneously into mice.

Figure 12 shows the effect of osteopontin gene copy number on pH of valves implanted subcutaneously into mice.

Figure 13 shows the effect of osteopontin gene copy number on carbonic anhydrase II expression at the site of valves implanted subcutaneously into mice.

Figure 14 shows the effect of osteopontin gene copy number on the number of multinucleate foreign body giant cells on valves implanted subcutaneously into mice.

Figure 15 shows the effect of osteopontin phosphorylation on valve mineralization *in vivo*.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to an effective method for the inhibition of ectopic calcification. Ectopic calcification commonly occurs in association with renal failure, cardiovascular disease, diabetes and the aging process. Ectopic calcification of the vasculature increases an individual's risk of myocardial infarction, ischemia, stroke, dissection after angioplasty and heart valve failure. Ectopic calcification of prosthetic implants, such as bioprosthetic heart valves, is the leading cause of implant failure. Therefore, the method

will reduce disease and death associated with ectopic calcification.

The method is based on the discovery that osteopontin is able to effectively and specifically inhibit ectopic calcification. Therefore, ectopic calcification can be prevented or treated by administering a therapeutically effective amount of osteopontin or a functional fragment thereof to an individual, either systemically or at the predicted or known sites of ectopic calcification. As osteopontin is normally found in calcified tissues and at the sites of ectopic calcification, it can be administered with minimal toxic or immunogenic side effects.

As used herein, the term "ectopic calcification" is intended to mean the abnormal deposition of calcium crystals at sites other than bones and teeth. Ectopic calcification results in the accumulation of macroscopic amorphous calcium phosphate and hydroxyapatite deposits in the extracellular matrix.

Ectopic calcification can occur in a variety of tissues and organs and is associated with a number of clinical conditions. For example, ectopic calcification can be a consequence of inflammation or damage to the affected tissues or can result from a systemic mineral imbalance. Commonly, ectopic calcification occurs in vascular tissue, including arteries, veins, capillaries, valves and sinuses. Inflammation or damage to the blood vessels can occur, for example, as a result of environmental factors such as smoking and high-fat diet. Inflammation or damage can also occur as a result of trauma to the vessels that results from injury, vascular surgery, heart surgery or angioplasty. Vascular

calcification is also associated with aging and with disease, including hypertension, atherosclerosis, diabetes, renal failure and subsequent dialysis, stenosis and restenosis.

Ectopic calcification also occurs in non-vascular tissues, such as tendons (Riley et al., Ann. Rheum. Dis. 55:109-115 (1996)), skin (Evans et al., Pediatric Dermatology 12:307-310 (1997)), sclera (Daicker et al., Opthalmologica 210:223-228 (1996) and myometrium (McCluggage et al., Int. J. Gynecol. Pathol. 15:82-84 (1996)), each of which is incorporated herein by reference. In diseases resulting in systemic mineral imbalance, such as renal failure and diabetes, ectopic calcification in visceral organs, including the lung, heart, kidney and stomach, is common (Hsu, Amer. J. Kidney Disease 4:641-649 (1997), incorporated herein by reference). Furthermore, ectopic calcification is a frequent complication of the implantation of biomaterials, prostheses and medical devices, including, for example, bioprosthetic heart valves (Vyavahare et al., Cardiovascular Pathology 6:219-229 (1997), incorporated herein by reference). The methods of the invention are applicable to ectopic calcification that occurs in association with all of these conditions.

The term "ectopic calcification" is not intended to refer to the calcification that normally occurs within the bone matrix during bone formation and growth. Ectopic calcification, as used herein, is also distinct from abnormal calcification that occurs in renal tubules and urine that results in the formation of primarily calcium oxalate-containing kidney stones.

As used herein, the term "inhibiting," in connection with inhibiting ectopic calcification, is intended to mean preventing, retarding, or reversing formation, growth or deposition of extracellular matrix hydroxyapatite crystal deposits.

As used herein, the term "osteopontin" is intended to mean a molecule that is able to inhibit ectopic calcification and that is recognizably similar to one or more molecules known in the art as osteopontin. Osteopontin is characterized as a phosphorylated sialoprotein having a predicted molecular weight of about 34 kDa. Due to high negativity, post-translational modifications and alternatively spliced isoforms, osteopontin has been reported to have an apparent molecular weight of between about 44 and 85 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Giachelli et al., Trends Cardiovasc. Med 5:88-95 (1995)). All of the post-translationally modified forms and alternatively spliced isoforms of osteopontin are included within the definition of osteopontin as used herein.

Osteopontin has been identified in various species, including rat (Oldbert et al., Proc. Natl., Acad. Sci. USA 83:8819-8823 (1986)); mouse (Craig et al., J. Biol. Chem. 264:9682-9689(1989)); human (Kiefer et al., Nucleic Acids Res. 17:3306 (1989) and Young et al. Genomics 7:491-502 (1990)); pig (Wrana et al., Nucleic Acids Res. 17:10119 (1989)); cow (Kerr et al., Gene 108:237-243 (1991)); rabbit (Tezuka et al., Biochem. Biophys. Res. Commun. 186:911-917 (1992)); and chicken (Moore et al., Biochemistry 30:2502-2508 (1991)), each of which is incorporated herein by reference. Osteopontin from these species and osteopontin homologs from other

vertebrates are included within the definition of osteopontin as used herein.

Osteopontin can be characterized by the presence of one or more domains that are conserved across known species. The conserved domains that characterize osteopontin include, for example, an N-terminal signal sequence, casein kinase II phosphorylation sites, an alternatively spliced domain, an Arg-Gly-Asp (RGD)-containing integrin-binding cell adhesion domain, an Asp-rich calcium binding domain, a calcium binding homology domain and two heparin binding homology domains (Giachelli et al., supra (1995)). Therefore, newly identified molecules that possess one or more of these characteristic features of osteopontin are also included within the definition of osteopontin.

Osteopontin is also known in the art as bone sialoprotein I, uropontin, secreted phosphoprotein I, 2ar, 2B7 and Eta 1 (Giachelli et al., supra (1995)). The molecules encompassed by all of these terms used in the art are included within the definition of osteopontin as used herein.

The nucleotide and deduced amino acid sequence for human osteopontin have been described by Kiefer et al., supra (1989), and are set forth herein as Figure 1 (SEQ ID NOS:1 and 2). The term osteopontin is intended to include, for example, polypeptides having substantially the same amino acid sequence as shown as SEQ ID NO:2 and encoded by substantially the same nucleotide sequence as shown as SEQ ID NO:1.

Modifications of osteopontin and its functional fragments that either enhance or do not greatly affect

the ability to inhibit ectopic calcification are also included within the term "osteopontin." Such modifications include, for example, additions, deletions or replacements of one or more amino acids from the native amino acid sequence of osteopontin with a structurally or chemically similar amino acid or amino acid analog. For example, the substitution of one or more phosphorylated amino acids, such as serine or threonine residues, by negatively charged amino acids, such as glutamic acid or aspartic acid, is contemplated. The substitution or addition of residues, such as kinase phosphorylation consensus sequences, that can be phosphorylated either *in vivo* or *in vitro* is also contemplated. Modifications of residues between the native sites of phosphorylation, such as to beneficially orient the phosphorylated residues to interact with hydroxyapatite or to reduce the distance between phosphorylation sites, is also contemplated. These modifications will either enhance or not significantly alter the structure, conformation or functional activity of the osteopontin or a functional fragment thereof.

Modifications that do not greatly affect the activity of osteopontin or its functional fragments can also include the addition or removal of sugar, phosphate or lipid groups as well as other chemical derivations known in the art. Additionally, osteopontin or its functional fragments can be modified by the addition of epitope tags or other sequences that aid in its purification and which do not greatly affect its activity.

As used herein, the term "functional fragment," in connection with osteopontin, is intended to mean a portion of osteopontin that maintains the ability of

osteopontin to inhibit ectopic calcification. A functional fragment can be, for example, from about 6 to about 300 amino acids in length, for example, from about 7 to about 150 amino acids in length, more preferably from about 8 to about 50 amino acids in length. If desired, a functional fragment can include regions of osteopontin with activities that beneficially cooperate with the ability to inhibit ectopic calcification. For example, a functional fragment of osteopontin can include sequences that promote the ingrowth of cells, such as endothelial cells and macrophages, at the site of ectopic calcification. Similarly, a functional fragment of osteopontin can include sequences, such as the RGD-containing domain, that beneficially promote cell adhesion and survival at the site of ectopic calcification.

As used herein, the term "individual" is intended to mean a human or other mammal, exhibiting, or at risk of developing, ectopic calcification. Such an individual can have, or be at risk of developing, for example, ectopic calcification associated with conditions such as atherosclerosis, stenosis, restenosis, renal failure, diabetes, prosthesis implantation, tissue injury or age-related vascular disease. The prognostic and clinical indications of these conditions are known in the art. An individual treated by a method of the invention can also be a candidate for, or have undergone, vascular surgery, including prosthetic valve replacement or angioplasty. An individual treated by a method of the invention can have a systemic mineral imbalance associated with, for example, diabetes, renal failure or kidney dialysis.

As used herein, the term "substantially the amino acid sequence," in reference to an osteopontin amino acid sequence or functional fragment thereof is intended to mean a sequence that is recognizably homologous to an osteopontin amino acid sequence and that inhibits ectopic calcification. For example, a sequence that is substantially the same as an osteopontin sequence can have greater than about 70% homology with an osteopontin sequence, preferably greater than about 80% homology, more preferably greater than about 90% homology.

As used herein, the term "prosthetic device" refers to a synthetic or biologically derived substitute for a diseased, defective or missing part of the body. As used herein, the term "bioprosthetic device" refers to a partially or completely biologically derived prosthetic device. Prosthetic devices are susceptible to ectopic calcification leading to premature failure, which can be inhibited by the methods of the invention. A prosthetic device can be implanted or attached at various sites of the body including, for example, the ear, eye, maxillofacial region, cranium, limbs and heart.

The methods of the invention can advantageously be used to prevent ectopic calcification of prosthetic heart valves, such as an aortic or atrioventricular valve, with or without a stent. Replacement heart valves can be made of a variety of materials, including metals, polymers and biological tissues, or any combination of these materials. Bioprosthetic valves include xenografted replacement valves from mammals, such as sheep, bovine and porcine, as well as human valves.

Bioprosthetic heart valves are commonly subjected to tissue fixation and can additionally be devitalized prior to implantation.

The invention provides a method of inhibiting ectopic calcification in an individual. The method consists of administering to the individual a therapeutically effective amount of osteopontin or a functional fragment thereof. The method is advantageous as it employs a molecule that normally occurs at the site of ectopic calcification as a therapeutic agent. Therefore, the method will result in minimal toxicity, immunogenicity and side effects.

Osteopontin can be prepared or obtained by methods known in the art including, for example, purification from an appropriate biological source or by chemical synthesis. An appropriate biological source of osteopontin can be tissues, biological fluids or cultured cells that contain or express osteopontin. The presence and abundance of osteopontin protein in a particular source can be determined, for example, using ELISA analysis (Min et al., Kidney Int. 53:189-93 (1998), incorporated herein by reference) or immunocytochemistry (O'Brien et al., Arterioscler. Thromb. 14:1648-1656 (1994), incorporated herein by reference).

Osteopontin has been determined to be present in or expressed by kidney cells, hypertrophic chondrocytes, odontoblasts, bone cells, bone marrow, inner ear and brain cells. Osteopontin is also found in biological fluids, including milk and urine. Osteopontin is also present in tumors, particularly metastatic tumors and is a component of kidney stones (Butler et al., In: Principles of Bone Biology, Bilezikian et al., eds.,

Academic Press, San Diego, pp. 167-181 (1996), incorporated herein by reference). Osteopontin is also produced by smooth muscle cells, macrophages and endothelial cells at the site of vascular lesions (O'Brien et al., Arterioscler. Thromb. 14:1648-1656 (1994), incorporated herein by reference). Therefore, osteopontin can be purified from any of these sources using biochemical purification methods known in the art.

Osteopontin can also be obtained from the secreted medium of cells of any of the above tissue lineages grown in culture. For example, osteopontin can be substantially purified from the conditioned medium of smooth muscle cell cultures as described by Liaw et al., Circ. Res. 74:214-224 (1994), incorporated herein by reference.

The nucleotide sequences of osteopontin from a variety of species are known, as described previously. Therefore, osteopontin or its functional fragments can also be recombinantly expressed by appropriate host cells including, for example, bacterial, yeast, amphibian, avian and mammalian cells, using methods known in the art. Methods for recombinant expression and purification of peptides in various host organisms are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainview, New York (1989) and in Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley and Sons, New York (1999), both of which are incorporated herein by reference. Methods for the recombinant synthesis and purification of osteopontin and exemplary functional fragments therefrom are described, for example, in Smith et al., J. Biol. Chem. 271:28485-28491 (1996), incorporated herein by reference.

Following recombinant synthesis and purification, osteopontin and its functional fragments can be modified in a physiologically relevant manner by, for example, phosphorylation, acylation or glycosylation, using enzymatic methods known in the art. A kinase that can be used to phosphorylate osteopontin or its functional fragments at biologically relevant sites is casein kinase II, as described in Example IV. Other serine-threonine kinases known in the art, such as protein kinase C can also be used to phosphorylate osteopontin or its functional fragments.

The methods of the invention can be practiced using osteopontin or any of its functional fragments that possess the activity of inhibiting ectopic calcification. Fragments of osteopontin are selected, produced by methods known in the art and screened as described herein to determine their ability to inhibit ectopic calcification.

Fragments of osteopontin can be produced, for example, by enzymatic or chemical cleavage of osteopontin. Methods for enzymatic and chemical cleavage and for purification of the resultant protein fragments are well known in the art (see, for example, Deutscher, Methods in Enzymology, Vol. 182, "Guide to Protein Purification," San Diego: Academic Press, Inc. (1990), which is incorporated herein by reference). As an example, osteopontin contains a thrombin cleavage site between Arg169 and Ser170. Either the N-terminal cleavage fragment or the C-terminal cleavage fragment of osteopontin can be used in the methods of the invention.

Fragments of osteopontin can also be produced by chemical or recombinant synthesis of peptides that

have substantially the sequence of osteopontin. For example, peptide libraries spanning overlapping sequences of osteopontin can be produced using methods known in the art and screened for their functional activity as described herein. Additionally, fragments corresponding to the N-terminal thrombin cleavage fragment or the C-terminal thrombin cleavage fragment of osteopontin can be recombinantly produced, as described by Smith et al., supra 271:28485-28491 (1996) and used in the methods of the invention.

As disclosed herein, osteopontin can inhibit ectopic calcification by directly adsorbing to and inhibiting apatite crystal growth and formation. Therefore, functional fragments of osteopontin can be selected based on their predicted ability to bind to calcium or calcium deposits. Regions that are contemplated to bind calcium include the aspartic acid rich sequence and the calcium binding homology domain. Therefore, a functional fragment of osteopontin can include, for example, substantially the sequence of the aspartic-rich calcium binding domain, DDMDDEDDDD (SEQ ID NO:3) or include substantially the sequence of the calcium binding homology domain, DWDSRGKDSYET (SEQ ID NO:4).

Additionally, as disclosed herein, phosphorylation can regulate the ability of osteopontin to inhibit ectopic calcification. Therefore, functional fragments of osteopontin can be selected by the presence of phosphorylation consensus sequences. A functional fragment of osteopontin can to chosen to include, for example, substantially the sequence of the casein kinase II phosphorylation consensus region, SGSSEEK (SEQ ID NO:5), or the C-terminal heparin binding homology domain

SKEEDKHLKFRISHELDSASSEVN (SEQ ID NO:6), which contains three conserved sites of serine phosphorylation. A functional fragment of osteopontin can alternatively or additionally include the alternatively spliced domain, NAVSSEETNDFKQE (SEQ ID NO:7), which contains two sites of serine phosphorylation. Additional sites of serine and threonine phosphorylation are described, for example, by Sorensen et al., Bioc. Biophys. Res. Comm. 198:200-205 (1994), incorporated herein by reference. A functional fragment of osteopontin can include one or several of these phosphorylated residues together with flanking amino acids.

Fragments of osteopontin having the ability to inhibit ectopic activity include regions of the molecule that are highly conserved among species. Regions within human osteopontin with high sequence conservation are presented, for example, in Giachelli et al., supra (1995). For example, a functional fragment can include the highly conserved sequence SDESHHSDESDE (SEQ ID NO:8). A functional fragment of osteopontin can also include the conserved cell adhesion domain, DGRGDSVAYG (SEQ ID NO:9) or the heparin binding homology domain RKKRSKKFRR (SEQ ID NO:10).

If desired, such as to optimize their functional activity, selectivity, stability or bioavailability, osteopontin or a functional fragment thereof can be modified to include D-stereoisomers, non-naturally occurring amino acids, and amino acid analogs and mimetics. Examples of modified amino acids are presented in Sawyer, Peptide Based Drug Design, ACS, Washington (1995) and Gross and Meienhofer,

The Peptides: Analysis, Synthesis, Biology, Academic Press, Inc., New York (1983), both of which are incorporated herein by reference.

If desired, one or more phosphorylated serine or threonine residues can be substituted by negatively charged amino acids, such as glutamic acid or aspartic acid. Such a modification can be advantageously made to reduce the susceptibility of osteopontin or a functional fragment to inactivation by phosphatases.

As disclosed herein, in addition to inhibiting ectopic calcification by directly adsorbing to and inhibiting apatite crystal growth and formation, osteopontin can also act by mediating an anti-calcific cellular response by promoting the accumulation and activation of carbonic anhydrase II (CAII) expressing multinucleated giant cells and macrophages, which are capable of acidifying the extracellular microenvironment and dissolving mineralized deposits. Through regulating CAII expression by multinucleated foreign body giant cells (FBGC) and macrophages, osteopontin can stimulate removal of calcium phosphate deposits.

The ability of osteopontin or a fragment selected and prepared as described above to inhibit ectopic calcification can be assayed by a variety of *in vitro* and *in vivo* assays known in the art or described herein. For example, as described in Example I, cultured vascular cells, such as bovine aortic smooth muscle cells, form calcified deposits in a time-dependent manner when treated with calcification medium containing β-glycerophosphate. Additionally, as described in Example III, human vascular smooth muscle cells form calcified deposits in the presence of elevated levels of

inorganic phosphate. Other culture systems for assaying the efficacy of osteopontin or a functional fragment thereof in inhibiting ectopic calcification can be determined by those skilled in the art. For example, osteopontin can be assayed using cells or tissues derived from other sites in the body where ectopic calcification occurs including, for example, viscera, skin, and endothelial cells.

The amount or extent of calcification prior to and following administering osteopontin or a functional fragment can be determined using such culture systems, either qualitatively by a visual or histochemical assessment, or by more quantitative methods. For example, calcified deposits can be detected visually as opaque areas by light microscopy, as black areas by von Kossa staining and as red areas by Alizarin Red S staining. The amount or extent of calcification can also be quantitatively assessed by the method described by Jono et al., Arterioscler. Thromb. Vasc. Biol. 17:1135-1142 (1997), incorporated herein by reference, or by using a commercially available colorimetric kit such as the Calcium Kit available from Sigma. Alternatively, the amount or extent of calcification can also be quantitatively assessed using known methods of atomic absorption spectroscopy.

As described in Examples I and III, the calcified deposits observed in cultured vascular smooth muscle cells, as assessed by histochemical, ultrastructural and electron diffraction analysis, can resemble the apatite deposits present at sites of ectopic calcification. Therefore, the ability of osteopontin or a functional fragment thereof to inhibit the deposition of calcium by cultured cells, in comparison with a

vehicle or protein control, is an accurate indicator of its ability to inhibit ectopic calcification in an individual.

The ability of osteopontin or a functional fragment thereof to inhibit ectopic calcification can also be tested in animal models known in the art to be reliable indicators of the corresponding human pathology. For example, ectopic calcification can be induced by the subcutaneous or circulatory implantation of bioprosthetic valves, such as porcine, sheep or bovine valves, into animals as described, for example, in Vyavahare et al., supra (1997). A reduction in the amount or rate of valve calcification by administration of osteopontin or a functional fragment can be detected, and is a measure of the functional activity of the preparation.

Similarly, animal models that are reliable indicators of human atherosclerosis, renal failure, hyperphosphatemia, diabetes, age-related vascular calcification and other conditions associated with ectopic calcification are known in the art. For example, topical and systemic calciphylaxis, calcinosis and calcergy, which are experimental models of ectopic calcification are described, for example, in Bargmann, J. Rheumatology 22:5-6 (1995), Lian et al., Calcified Tissue International, 35:555-561 (1983) and Boivin et al., Cell and Tissue Res. 247:525-532 (1987). An experimental model of calcification of the vessel wall is described, for example, by Yamaguchi et al., Exp. Path. 25:185-190 (1984).

A preferred animal model for examining ectopic calcification and the effect of osteopontin preparations is an osteopontin-deficient mouse, described by Liaw et

al., J. Clin. Invest. 101:1468-1478 (1998), incorporated herein by reference, in which, as described in Example V, ectopic calcification is enhanced compared to wild-type control animals.

Medical imaging techniques known in the art, such as magnetic resonance imaging, X-ray imaging, computed tomography and ultrasonography, can be used to assess the efficacy of osteopontin or a functional fragment thereof in inhibiting ectopic calcification in either a human or an animal. For example, the presence and extent of calcium deposits within vessels can be determined by the intravascular ultrasound imaging method described by Fitzgerald et al., Circulation 86:64-70 (1994), incorporated herein by reference. A decrease in the amount or extent of ectopic calcification can readily be identified and is indicative of the therapeutic efficacy of osteopontin or a functional fragment thereof.

Osteopontin or its functional fragments, assayed for their functional activity as described above, are administered to an individual in a therapeutically effective amount to inhibit ectopic calcification. Appropriate formulations, dosages and routes of delivery for administering osteopontin or a functional fragment are well known to those skilled in the art and can be determined for human patients, for example, from animal models as described previously. The dosage of osteopontin or a functional fragment thereof required to be therapeutically effective can depend, for example, on such factors as the extent of calcification, the site of calcification, the route and form of administration, the bio-active half-life of the molecule being administered, the weight and condition of the individual, and previous or concurrent therapies. The appropriate amount

considered to be a therapeutically effective dose for a particular application of the method can be determined by those skilled in the art, using the guidance provided herein. One skilled in the art will recognize that the condition of the patient needs to be monitored throughout the course of therapy and that the amount of the composition that is administered can be adjusted accordingly.

For treating humans, a therapeutically effective amount of osteopontin or its functional fragments can be, for example, between about 10 µg/kg to 500 mg/kg body weight, for example, between about 0.1 mg/kg to 100 mg/kg, or between about 1 mg/kg to 50 mg/kg, depending on the treatment regimen. For example, if osteopontin or a functional fragment is administered several times a day, or once a day, or once every several days, a lower dose would be needed than if osteopontin or a functional fragment were administered only once, or once a week, or once every several weeks. Similarly, formulations that allow for timed-release of osteopontin would provide for the continuous release of a smaller amount of osteopontin than would be administered as a single bolus dose.

Osteopontin or a functional fragment can be delivered systemically, such as intravenously or intraarterially, to inhibit ectopic calcification throughout the body. Osteopontin or a functional fragment can also be administered locally at a site known to contain or predicted to develop ectopic calcification. Such a site can be, for example, an atherosclerotic plaque, a segment of artery undergoing angioplasty or the site of prosthetic implantation. Appropriate sites for administration of osteopontin and its functional

fragments can be determined by those skilled in the art depending on the clinical indications of the individual being treated and whether or not the individual is concurrently undergoing invasive surgery.

Administration of osteopontin or a functional fragment can be achieved using various formulations of osteopontin. If desired, osteopontin can be administered as a solution or suspension together with a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be, for example, water, sodium phosphate buffer, phosphate buffered saline, normal saline or Ringer's solution or other physiologically buffered saline, or other solvent or vehicle such as a glycol, glycerol, an oil such as olive oil or an injectable organic ester.

A pharmaceutically acceptable carrier can additionally contain physiologically acceptable compounds that act, for example, to stabilize or increase the absorption of osteopontin or a functional fragment. Such physiologically acceptable compounds include, for example, carbohydrates such as glucose, sucrose or dextrans; antioxidants such as ascorbic acid or glutathione; chelating agents such as EDTA, which disrupts microbial membranes; divalent metal ions such as calcium or magnesium; low molecular weight proteins; lipids or liposomes; or other stabilizers or excipients. Osteopontin can also be formulated with a material such as a biodegradable polymer or a micropump that provides for controlled slow release of the molecule. Additionally, osteopontin can be formulated with a molecule, such as a phosphatase inhibitor, that reduces or inhibits dephosphorylation of osteopontin.

Osteopontin or a functional fragment can also be expressed from cells that have been genetically modified to express the protein. Expression of osteopontin from a genetically modified cell provides the advantage that sustained localized or systemic expression of the protein can occur, thus obviating the need for repeated administrations.

Methods for recombinantly expressing proteins in a variety of mammalian cells for therapeutic purposes are known in the art and are described, for example, in Lee et al., Transfusion Medicine II 9:91-113 (1995), which is incorporated herein by reference. Types of cells that are particularly amenable to genetic manipulation include, for example, hematopoietic stem cells, hepatocytes, vascular endothelial cells, keratinocytes, myoblasts, fibroblasts and lymphocytes.

A nucleic acid encoding osteopontin or a functional fragment can be operatively linked to a promoter sequence, which can provide constitutive or, if desired, inducible expression of appropriate levels of the encoded protein. Suitable promoter sequences for a particular application of the method can be determined by those skilled in the art and will depend, for example, on the cell type and the desired osteopontin expression level.

The nucleic acid encoding osteopontin or a functional fragment thereof can be inserted into a mammalian expression vector and introduced into cells by a variety of methods known in the art (see, for example, Sambrook et al., supra (1989); and Ausubel et al., supra (1994)). Such methods include, for example, transfection, lipofection, electroporation and infection

with recombinant vectors. Infection with viral vectors such as retrovirus, adenovirus or adenovirus-associated vectors is particularly useful for genetically modifying a cell. A nucleic acid molecule also can be introduced into a cell using known methods that do not require the initial introduction of the nucleic acid sequence into a vector.

In one embodiment of the invention, a prosthetic device can be contacted with osteopontin or a functional fragment thereof. Contacting a prosthetic device with osteopontin or a functional fragment will effectively prevent or reduce ectopic calcification of the prosthetic device, preventing failure of the device and the need for premature replacement. The prosthetic device can be contacted with osteopontin or a functional fragment either prior to, during or following implantation into an individual, as needed.

Osteopontin or a functional fragment can contact a prosthetic device by attaching the molecule either covalently or non-covalently to the prosthetic device. An appropriate attachment method for a particular application of the method can be determined by those skilled in the art. Those skilled in the art know that an appropriate attachment method is compatible with implantation of the prosthetic device in humans and, accordingly, will not cause unacceptable toxicity or immunological rejection. Additionally, an appropriate attachment method will enhance or not significantly reduce the ability of osteopontin or a functional fragment thereof to inhibit ectopic calcification of the prosthetic device and the surrounding tissue.

Methods for covalently attaching proteins to polymers, metals and tissues are known in the art. For example, osteopontin can be attached to the prosthetic device using chemical cross-linking. Chemical cross-linking agents include, for example, glutaraldehyde and other aldehydes. Cross-linking agents that link osteopontin or a functional fragment thereof to a prosthetic device through either a reactive amino acid group, a carbohydrate moiety, or an added synthetic moiety are known in the art. Such agents and methods are described, for example, in Hermason, Bioconjugate Techniques, Academic Press, San Diego (1996), which is incorporated herein by reference. These methods can be used to contact a prosthetic device with a therapeutically effective amount of osteopontin or a functional fragment thereof.

Osteopontin can also be attached non-covalently to the prosthetic device by, for example, adsorption to the surface of the prosthetic device. A solution or suspension containing osteopontin or a functional fragment thereof, together with a pharmaceutically acceptable carrier, if desired, can be coated onto the prosthetic device in a therapeutically effective amount.

To provide sustained delivery of osteopontin or a functional fragment, a prosthetic device can also be contacted with osteopontin or a functional fragment thereof produced by cells attached to the prosthetic device. Such cells can be seeded onto the prosthetic device and expanded either *ex vivo* or *in vivo*. Appropriate cells include cells that normally produce and secrete osteopontin including, for example, macrophages, smooth muscle cells or endothelial cells. Additionally, cells that have been genetically modified to produce

osteopontin or a functional fragment thereof including, for example, endothelial cells and fibroblasts, can be attached to the prosthetic device. The cells that are attached to the prosthetic device are preferably either derived from the individual receiving the prosthetic implant, or from an immunologically matched individual to reduce the likelihood of rejection of the implant.

The ability of osteopontin or a functional fragment that contacts a prosthetic device to inhibit ectopic calcification can be determined by various methods known in the art. One such method is to implant the prosthetic device into animals and measure calcium deposition, as described in Example V, in response to administration of osteopontin or a functional fragment thereof. Either a decrease in the rate or the amount of calcium deposition at the site of the explant is indicative of the therapeutic efficacy of the composition.

The invention provides a method of treating or inhibiting ectopic calcification by administering to an individual cells that acidify the local microenvironment, whereby the cells are targeted to the site of ectopic calcification. Multinucleate foreign body giant cells and macrophages are examples of cells capable of acidifying the local microenvironment. Therefore, the invention provides a method of treating or inhibiting ectopic calcification by administering to an individual macrophages, whereby the macrophages are targeted to the site of ectopic calcification. In addition, the invention provides a method of treating or inhibiting ectopic calcification by administering to an individual multinucleate foreign body giant cells, whereby the

multinucleate foreign body giant cells are targeted to the site of ectopic calcification.

As used herein, the term "local microenvironment" refers to the extracellular space that can be acidified either by recruitment and/or differentiation of acid producing cells or by promotion of proton production and extracellular release of cell protons. For example, osteoclasts and macrophages express carbonic anhydrase II, an enzyme that promotes the hydrolysis of carbon dioxide to carbonic acid. Carbonic acid serves as an intracellular source of protons that are exported from the cell via a vacuolar H^+-ATPase, thereby reducing local pH and promoting mineral dissolution. Therefore, the invention provides a method of treating or inhibiting ectopic calcification via osteopontin-mediated expression of carbonic anhydrase II by macrophages and multinucleate foreign body giant cells.

As disclosed herein, osteopontin regulates ectopic mineralization not only by directly binding to apatite crystal surfaces and inhibiting crystal growth (see Example II), but also indirectly by regulating mineral loss (Example V). Specifically, osteopontin acts as a mediator of an anti-calcific cellular response by promoting macrophage recruitment, increased carbonic anhydrase II expression and extracellular acidification.

The cells capable of acidifying the local microenvironment such as macrophages and multinucleate foreign body giant cells are targeted to the site of ectopic calcification. For example, macrophages and multinucleate foreign body giant cells can be attached to the prosthetic device. For example, macrophages and

multinucleate foreign body giant cells can be seeded onto the bioprosthetic heart valve and expanded *ex vivo* or *in vivo* as described above. Additionally, cells that have been genetically modified to express carbonic anhydrase II, or a functional fragment thereof, can be attached to the prosthetic device. A functional fragment of carbonic anhydrase II retains osteopontin-mediated regulation as well as catalytic activity. As described above for osteopontin-producing cells, the carbonic anhydrase II-producing cells are preferably derived from the individual receiving the prosthetic implant or from an immunologically matched individual to reduce the likelihood of implant rejection. In addition, macrophages and multinucleate foreign body giant cells can be administered to the site of ectopic calcification in an individual either directly or systemically. If administered systemically, the administered macrophages and multinucleate foreign body giant cells can be targeted to the site of ectopic calcification. For example, precoating a bioprosthetic device with osteopontin or osteopontin antibody is one way to target systemically administered macrophages and multinucleate foreign body giant cells to a site of ectopic calcification to acidify the local microenvironment.

As described in Example V, acidification of the local microenvironment can also be achieved by administering osteopontin, which can regulate carbonic anhydrase II expression by macrophages and multinucleate foreign body giant cells. Methods of administering osteopontin to an individual are described in detail above. The expression level of osteopontin required to stimulate carbonic anhydrase II expression can be significantly lower than the expression level required to directly inhibit hydroxyapatite growth. Therefore, a

therapeutically effective amount for stimulating carbonic anhydrase II can be within the range of about $1x10^{-12}$ M to $5x10^{-7}$ M . Therapeutically effective amounts of osteopontin sufficient to stimulate expression of carbonic anhydrase II can also be within the range of about $1x10^{-12}$ M to $1x10^{-7}$ M, $1x10^{-12}$ M to $5x10^{-8}$ M, $1x10^{-12}$ M to $1x10^{-8}$ M, $1x10^{-12}$ M to $5x10^{-9}$ M, $1x10^{-12}$ M to $1x10^{-9}$ M, $1x10^{-12}$ M to $5x10^{-10}$ M, $1x10^{-12}$ M to $5x10^{-10}$ M, or $1x10^{-12}$ M to $5x10^{-11}$ M.

Thus, the invention provides a method of treating or inhibiting ectopic calcification by administering to an individual cells that acidify the local microenvironment. The invention further provides a method of treating or inhibiting ectopic calcification in an individual comprising promoting recruitment of acid producing cells to a site of ectopic calcification by administering osteopontin. The invention also provides a method of treating or inhibiting ectopic calcification in an individual comprising promoting differentiation of acid producing cells at a site of ectopic calcification by administering osteopontin. The invention further provides a method of treating or inhibiting ectopic calcification in an individual comprising promoting recruitment and differentiation of acid producing cells to a site of ectopic calcification by administering osteopontin. As described in Experiment V, mice carrying one copy of the osteopontin gene express high enough levels of osteopontin to promote formation of carbonic anhydrase II positive cells in response to ectopic mineralization (see Figure 13). Macrophages and multinucleate foreign body giant cells are carbonic anhydrase II-expressing cells derived from a similar hematopoietic origin and shown herein to be localized

adjacent to mineralized implants in mice carrying one copy of the osteopontin gene.

As described in Example V, osteopontin can control mineral resorption by recruiting acid producing cells to the implantation site of gutaraldehyde-fixed aortic valve (GFAV) leaflets in mice. For example, as demonstrated by immunostaining, osteopontin can mediate the recruitment of macrophages and promote CAII-positive cell formation (see Example V). As disclosed herein, macrophages and multinucleate foreign body giant cells are examples of cells expressing carbonic anhydrase II that are regulated by osteopontin. Methods of administering osteopontin are described in detail above.

The invention also provides a method of treating or inhibiting ectopic calcification in an individual by increasing the expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin. As disclosed herein, osteopontin controls mineral resorption by regulating the differentiation and activity of carbonic anhydrase II expressing cells, including macrophages and multinucleate foreign body giant cells (see Example V).

It is understood that modifications that do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Calcification of cultured bovine vascular cells

This example demonstrates that calcium deposition by cultured bovine aortic smooth muscle cells is a credible model of ectopic calcification. Methods for inducing physiologically relevant calcification are described. These methods can be used to assay preparations of osteopontin and fragments thereof for their ability to inhibit ectopic calcification.

Culture of bovine aortic smooth muscle cells

BASMCs were obtained by a modification of the explant method originally described by Ross et al., J. Cell Biol., 50:172-186 (1971), which is incorporated herein by reference. Briefly, medial tissue was separated from segments of bovine thoracic aorta. Small pieces of tissue (1 to 2 mm^3) were loosened by a one-hour incubation in DMEM containing 4.5 g/L of glucose supplemented with 165 U/ml collagenase type I, 15 U/ml elastase type III and 0.375 mg/mL soybean trypsin inhibitor at 37°C. Partially digested tissues were placed in 6-well plates and cultured for several weeks in DMEM containing 4.5 g/L of glucose supplemented with 20% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells that had migrated from the explants were collected and maintained in growth medium (DMEM containing 15% FBS and 10 mM sodium pyruvate supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin). To confirm that the cells isolated from bovine aortic wall were

vascular smooth muscle cells, α-smooth muscle actin, vimentin, and calponin levels were examined by immunofluorescence microscopy.

For immunofluorescence microscopy, BASMCS were cultured on 10-well heavy Teflon-coated microscope glass slides (Cel-Line Associates Inc., USA) for 24 hours, fixed with cold methanol, blocked with PBS containing 2% BSA and 10% normal rabbit serum, and treated with monoclonal anti-α-smooth muscle actin antibody (1A4, Sigma) and monoclonal anti-vimentin antibody (V9, Dako) diluted with PBS containing 2% BSA 1:50 and 1:25, respectively. Monoclonal anti-calponin antibody (CALP), Frid et al., Dev. Biol., 153:185-193 (1992), was used without dilution. As a secondary antibody, FITC-conjugated rabbit anti-mouse IgG was used after dilution with PBS 1:30. Mouse non-immune IgG was used as a control for the primary antibody.

Greater than 95% of the cells obtained as described above were stained with α-smooth muscle actin, vimentin, and calponin antibodies in a filamentous pattern, indicating that the cultured cells were of vascular smooth muscle origin. For all experiments, cells were used between passages 2 and 5.

Calcium deposition by bovine aortic smooth muscle cells

In order to examine calcification by cultured BASMC smooth muscle cells, calcification was induced by the method described by Shioi et al., Arterioscler Thromb Vasc Biol., 15:2003-2009 (1995), which is incorporated herein by reference. Briefly, BASMC were cultured in growth medium for 4 days, and then switched to calcification medium (DMEM (high glucose, 4.5 g/L)

containing 15% FBS and 10 mM sodium pyruvate in the presence of 10 mM of β-glycerophosphate (unless otherwise stated), 10-7 M insulin, and 50 µg/ml of ascorbic acid, supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin) for 10 days. The medium was replaced with fresh medium twice a week. In the time course experiments, the beginning day of culture in calcification medium was defined as day O.

Calcification was assessed by a modification of the method described by Jono et al., Arterioscler. Thromb. Vasc. Biol. 17:1135-1142 (1997) which is incorporated herein by reference. Briefly, the cultures were decalcified with 0.6 N HCl for 24 hours. The calcium content of the HCl supernatant was determined colorimetrically by the o- cresolphthalein complexone method (Calcium Kit, Sigma). After decalcification, the cultures were washed with phosphate-buffered saline (PBS) and solubilized with 0.1 N NaOH/O. 1% sodium dodecyl sulfate(SDS). Total protein content was measured with a Bio-Rad Protein Assay Kit (Bio-Rad). The calcium content of the cell layer was normalized to protein content. Phosphorus and calcium concentrations in the culture medium were measured by the phosphomolybdate complex method (Phosphorus Kit, Sigma) and the o-cresolphthalein complexone method (Calcium Kit, Sigma), respectively. Values were expressed as the mean +/- SEM, n=3.

BASMC treated with calcification medium containing β-glycerophosphate initiated calcium-containing mineral deposition in a time-dependent manner over the course of 14 days. In contrast, BASMC cultured in growth medium lacking β-glycerophosphate did not calcify. Addition of β-glycerophosphate resulted in an

increased phosphorus concentration which correlated positively with calcium deposition in the cell layer. Conversely, calcium concentration decreased in the culture medium as the cell layer became calcified.

The effects of β-glycerophosphate on calcium deposition, phosphorus concentration and calcium concentration in the medium were dose-dependent. Calcium deposition depended on the initial concentration of β-glycerophosphate and was half-maximal at ~4 mM β-glycerophosphate. Phosphorus concentration in the culture medium increased with increasing concentrations of β-glycerophosphate over the range of 0 to 10 mM. Calcium deposition in the culture medium was inversely proportional to calcium deposition in the cell layer.

The observed calcification was not due to spontaneous precipitation of mineral from the media as supplementation of the culture media with up to 10 mM inorganic phosphate failed to form calcified deposits in the absence of cells. Nor did addition of calcification media to endothelial cell cultures induce mineralization.

These results indicate that the calcification of BASMC under conditions which elevate inorganic phosphate in the media is a specific, cell- and matrix-mediated event.

Morphology of BASMC calcification

To determine whether the calcification process in the BASMC cultures represented a physiologic-type of mineralization, histochemical, ultrastructural, and electron diffraction analyses were performed.

Mineral deposition by BASMC cultures was assessed histochemically by von Kossa staining (30 minutes, 5% silver nitrate) and light microscopy using the method described by Mallory, F.B., in Pathological Techniques, Second Edition, Philadelphia, WB Saunders Co., p. 152 (1942), which is incorporated herein by reference). The expression of alkalinephosphatasee was visualized by incubating citrate-acetone-formaldehyde fixed cells at room temperature for 15 minutes with Naphthol AS-BI Alkaline Solution (Sigma).

For ultrastructural examination by transmission electron microscopy (TEM), BASMC cells grown on plastic were fixed overnight in an aldehyde solution containing 1% glutaraldehyde and 1% paraformaldehyde buffered with 0.1 M sodium cacodylate buffer at pH 7.2. The cultures were then washed with 0.1 M sodium cacodylate buffer alone, dehydrated in a graded series of ethanol solutions, and infiltrated and embedded in either Taab epoxy resin or LR White acrylic resin (Marivac, Nova Scotia, Canada). The resins were polymerized for 2 days at 55°C. Samples destined for epoxy embedding were also post-fixed with potassium ferrocyanide-reduced 4% osmium tetroxide to provide additional membrane contrast in the electron microscope.

For mineral analyses by selected-area electron diffraction, other cultures were treated nonaqueously by fixing only with 100% ethanol, followed by direct embedding in resin without further processing. One micrometer-thick survey sections were prepared from various regions of the cultures and stained with Toluidine blue for examination by light microscopy. Thin sections (80-100 nm) of selected regions were then cut using a diamond knife on a Reichert Ultracut E microtome

and placed on Formvar-coated nickel grids evaporated with carbon. Grid-mounted sections were stained briefly with ethanolic uranyl acetate and lead citrate and examined using a JEOL JEM 1200EX transmission electron microscope operating at 60 kV. Anhydrously treated samples left unstained were used for selected-area electron diffraction using a 100 μM diffraction aperture and a camera length of 80 cm. Diffraction patterns were analyzed and compared with synthetic apatite standards and powder diffraction files as previously reported for bone mineral (Landis et al., J. Ultrastruc. Res., 63:188-223 (1978), incorporated herein by reference).

By light microscopy, BASMC cultures grown in growth medium showed areas of monolayer and multilayered growth typical for these types of cells. Following treatment with calcification medium for 10 days, the cultures showed extensive deposition of mineral, predominantly in multilayered areas. Von Kossa staining confirmed the presence of phosphate-containing mineral in these cultures. The calcification was most often observed in the extracellular matrix between cells, and was typically more pronounced at the basal aspect of the culture. The BASMCs in these calcified cultures were also positive for alkaline phosphatase activity.

At 14 days of culture (10 days with β-glycerophosphate), BASMC were monolayered or multilayered and at some locations formed nodules of cells. Ultrastructurally, where multilayered or nodular in appearance, the cells were associated with abundant extracellular matrix rich in collagen fibrils. At sites of this extracellular matrix accumulation, cells exhibited well-developed organelles typically associated with protein synthesis and secretion. A prominent

cytoskeleton was evidenced by an extensive network of intracellular microfilaments, most likely composed of actin.

Whereas cells cultured without β-glycerophosphate showed no evidence of extracellular matrix calcification, those cultured with the added organic phosphate source showed several morphologically distinct forms of calcification associated with the cell layer. These included roughly spherical aggregates of calcified collagen fibrils, nodular deposits with increased mineral density at the periphery, and more diffuse calcification involving both the intra- and interfibrillar compartments of the extracellular matrix. At these latter sites, crystals having somewhat larger dimensions were observed to extend from one collagen fibril to another. Membrane-bounded matrix vesicles were also found in the extracellular matrix. Selected-area electron diffraction of anhydrously treated and unstained tissue sections of BASMC cultures containing calcified deposits identified the mineral phase as apatite, showing prominent diffraction reflections (from lattice planes 002, 211, 112, 300) whose indices were characteristic for this type of mineral.

Alkaline phosphatase is required for normal bone mineralization (Whyte et al. Endocr. Rev., 15:439-461 (1994)) and has been shown to be required for calcification of osteoblast and cartilage cell cultures in response to β-glycerophosphate (Tenenbaum et al., Bone Mineral, 2:13-26 (1987)). To determine whether alkaline phosphatase was required for calcification in BASMCs under the conditions used in these studies, cultures were treated with the alkaline phosphatase inhibitor levamisole, or with vehicle alone. Calcium deposition in

BASMC cultures was dose-dependently inhibited by levamisole. Half-maximal inhibition was observed at 5 x 10^{-5} M levamisole. Vehicle treatment had no effect. Levamisole treatment was associated with a decrease in phosphorus concentration and maintenance of high calcium concentration in the culture medium.

These results indicate that calcification of the matrix deposited by BASMC cultures resembles the mineralization observed at sites of ectopic calcification in regard to mineral type (apatite) and the ultrastructure of the calcified deposits. For example, mineralization occurred predominantly in association with extracellular matrix collagen fibrils and matrix vesicles. Similar vesicular structures have been reported in calcified atherosclerotic plaques in association with elevated alkaline phosphatase activity (Kim et al., Fed Proc, 35:156-162 (1976)). Additionally, the nodular calcifications present in the calcifying BASMC cultures indicate spherulitic crystal growth which is a common observation in calcified atherosclerotic plaques and valves (Kim et al., Fed Proc, 35:156-162 (1976)).

Therefore, the calcifying BASMC cultures used in these studies are able to create an extracellular milieu capable of mineralization similar to the mineralization observed in calcified vascular tissues *in vivo*, supporting their use as a model of ectopic calcification.

EXAMPLE II

Osteopontin inhibits BASMC calcification

This example demonstrates that osteopontin inhibits BASMC calcification *in vitro*, which is a credible model of ectopic calcification *in vivo*. Therefore, osteopontin will be a therapeutically effective inhibitor of ectopic calcification.

Rat osteopontin was purified from the conditioned medium of rat neonatal smooth muscle cell cultures as described by Liaw et al., supra 74:214-224 (1994), which is incorporated herein by reference. This preparation was judged to be >95% pure based on Coomassie staining and N-terminal sequence analysis.

To examine the effect of osteopontin on BASMC-mediated calcification *in vitro*, soluble osteopontin or vehicle alone (0.1 mM sodium citrate) was added to the calcifying BASMC cultures. As shown in Figure 2a, osteopontin at 0.05, 0.5 and 5 µg/ml dose-dependently inhibited calcification assessed at 10 days. For example, 0.5 µg/ml of osteopontin inhibited calcification by approximately 90%, and 5 µg/ml osteopontin almost completely inhibited calcification. In contrast, vehicle alone had no effect (Figure 2a). Therefore, low concentrations of exogenously applied osteopontin profoundly inhibits extracellular mineralization in a calcifying vascular cell culture system.

To exclude the possibility that contaminants in the osteopontin preparation were responsible for the observed inhibitory effect, immunodepletion experiments were performed. Medium containing 10 µg/ml osteopontin was mixed with 20 mg/ml anti-osteopontin (OP-199) or normal goat IgG, prepared by the method described by Liaw et al., supra (1994) and incubated for 1 hr at room temperature. 250 mg protein-A-sepharose was added and incubated for 1 hr at room temperature. The antibody-protein A sepharose complexes were removed by centrifugation, and the remaining supernatant diluted twenty-fold for use in the calcification studies. Unpaired Student's t test was employed to compare groups and a probability value (p) value less than 0.05 was considered significant.

Medium containing 0.5 ug/ml rat osteopontin inhibited calcification of the cultures by 18 fold (5.05 ± 0.25 µmole/mg for vehicle-treated versus 0.33 ± 0.06 µmole/mg for osteopontin-treated BASMC p=0.0023). Immunodepletion of the osteopontin solution with osteopontin antibody significantly reduced its inhibitory activity (0.33 ± 0.06 µmole/mg for nonimmunodepleted sample versus 2.60 ± 0.43 µmole/mg for anti-osteopontin depleted samples, p=0.0338). In contrast, immunodepletion with normal goat IgG did not affect the inhibitory activity of the rat osteopontin solution (0.49 ± 0.10 µmole/mg for normal goat IgG-treated versus 0.33 ± 0.06 µmole/mg with no immunodepletion, p=0.2480).

These results confirm that the observed inhibition of BASMC-mediated calcification by the osteopontin preparation was specifically due to osteopontin, rather than due to a contaminant.

To determine the specificity and uniqueness of osteopontin's effects, two additional noncollagenous extracellular matrix RGD-containing molecules with limited structural and functional homology to osteopontin, vitronectin and fibronectin, were tested for their ability to inhibit BASMC-mediated calcification. Rat plasma vitronectin (Sigma Immunochemicals, USA) and bovine fibronectin (TELIOS Pharmaceutical Inc., USA) were resuspended in PBS at a concentration of 0.5 mg/ml and stored frozen until use. As shown in Figure 2b, vitronectin (VN) and fibronectin (FN), at equimolar concentrations as were effective for osteopontin, were unable to inhibit calcium deposition. Therefore, the effect of osteopontin on inhibiting vascular calcification is highly specific. Furthermore, these results indicate that the capacity of osteopontin to modulate mineralization are unrelated to its RGD-dependent cell adhesive functions.

Mechanism of osteopontin inhibition

The mechanism by which osteopontin inhibited calcification was tested. One possibility was that osteopontin might function in a manner similar to levamisole by affecting alkaline phosphatase activity, thereby inhibiting production of inorganic phosphate from β-glycerophosphate and preventing calcium phosphate deposition.

For cellular alkaline phosphatase activity measurements, cells were cultured in calcification medium in the presence of various concentrations of osteopontin. Cells were washed three times with PBS and cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for

alkaline phosphatase activity by the method described by Bessey et al., J. Biol. Chem. 164:321-329 (1946), which is incorporated herein by reference. One unit was defined as the activity producing 1 nmol of p-nitrophenol within 1 minute. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad). The data were normalized to the protein content of the cell layer.

Treatment with osteopontin did not affect the alkaline phosphatase activity of BASMC cultures, as shown in Figures 3a and 3c. The addition of osteopontin also did not reduce the phosphorus concentration of the medium. In contrast, levamisole dose-dependently inhibited BASMC alkaline phosphatase activity (Figure 3b) and reduced the phosphorus concentration in the culture medium. These results demonstrate that osteopontin does not act by inhibiting alkaline phosphatase activity.

The possibility that osteopontin chelates or sequesters calcium in the culture media to prevent mineralization was also tested. The initial calcification medium was supplemented with increasing concentrations of calcium in the presence of osteopontin or vehicle alone. Cultures were then allowed to calcify in the presence or absence of osteopontin over a 10 day period. As shown in Figure 4a, increasing the calcium content of the medium was able to overcome the inhibitory effect of osteopontin on calcium deposition, allowing more mineral to be deposited in the cell layer. Consistent with this, a decrease in the phosphorus content (from 8.2 mM to 7.3 mM) of the culture media was noted (Figure 4b).

The calcium content of the media at the end of the 10 day period in the presence of osteopontin was also

measured. If osteopontin acted by sequestering calcium, it was expected that either a constant or an increasing amount of calcium would be observed in the medium, reflecting retention of calcium in the medium by osteopontin binding. However, the opposite was observed. Calcium concentration in the culture medium was decreased at the end of the 10 day period compared to initial calcium concentrations, and correlated inversely with calcium deposition (compare Figures 4a and 4c). Therefore, the inhibitory effect of osteopontin on mineralization is calcium dependent (i.e. decreased by increasing calcium concentrations), but is not attributable to chelation of the calcium available in the medium. This observation is consistent with the known calcium binding properties of osteopontin. It has been shown that about 50 molecules of calcium can be bound by osteopontin at physiological calcium concentrations (Chen et al., J. Biol. Chem. 267:24871-24878 (1992)). Hence it would require about 40 μM osteopontin (2.7 mg/ml) to chelate 2 mM calcium, which is more than 5000 times the amount of osteopontin (0.5 μg/ml) demonstrated to be effective in inhibiting vascular calcification in the assays described herein.

The ultrastructural localization of endogenous and exogenous osteopontin in the BASMC cultures was also determined using immunogold labeling to further characterize the mechanism of osteopontin inhibition of vascular calcification. BASMC were cultured in calcification media for 7 days to allow mineralization to begin. Purified rat osteopontin (0.5 μg/ml) was then added until day 10. Cultures were preserved using aldehyde fixative followed by embedding in LR White acrylic resin for immunocytochemistry. Post-embedding immunolabeling was performed using osteopontin antibody

(OP-199) and protein A-colloidal gold complex as described by McKee et al., Microscop. Res. And Tech., 33:141-164 (1996), which is incorporated herein by reference. Briefly, thin (80 nm) sections of the cultures were placed on nickel grids and incubated for 5 min with 1% ovalbumin in PBS, followed by incubation with primary antibody for 1 hr, rinsing with PBS, blocking again with ovalbumin, and then exposure to protein A-gold complex for 30 min. After final rinsing with distilled water, grids were air dried and conventionally stained with uranyl acetate and lead citrate and viewed by transmission electron microscopy. The specificity of the OP- 199 antibody has been shown previously by Western blotting (Liaw et al., supra, (1994)) and by incubations using pre-immune serum and protein A-gold complex alone.

For these immunogold labeling studies, osteopontin was omitted (vehicle alone) or added on day 7 following initiation of mineralization with β-glycerophosphate. Under these conditions, exogenously applied osteopontin (0.5 ug/ml) was still able to inhibit BASMC culture calcification by 50% at day 10. A low level of endogenous osteopontin was found in untreated, mineralizing cultures, typically in a diffuse pattern in the mineralized areas. In contrast, in osteopontin-treated cultures, gold particles were abundant at sites of calcification, typically accumulating at the margins of small calcified masses or associating with individual crystal profiles. No gold particles were observed when pre-immune serum and protein A-gold complex alone were used as controls, indicating that a direct interaction of osteopontin with the growing apatite crystals is required for its inhibitory function. Osteopontin was not observed to be associated with unmineralized matrix or cells.

The results described above demonstrate that osteopontin is able to inhibit physiological calcification mediated by vascular cells at low concentrations by direct binding of osteopontin to apatite crystal surfaces and inhibition of crystal growth. Therefore, osteopontin will be therapeutically useful in preventing and treating ectopic calcification.

EXAMPLE III

Calcification of cultured human vascular cells

This example shows that calcium deposition by cultured human smooth muscle cells in the presence of elevated inorganic phosphate is a credible model of ectopic calcification. Methods for inducing physiologically relevant calcification are described. These methods can be used to assay preparations of osteopontin and fragments thereof for their ability to inhibit ectopic calcification.

The normal adult range of serum inorganic phosphate concentration is about 1.0-1.5 mM. A high serum phosphate level, or hyperphosphatemia, occurs in association with various disease states including, for example, chronic renal failure and subsequent kidney dialysis. In such disease states the serum inorganic phosphate levels can typically exceed 2 mM. In order to model ectopic calcification associated with hyperphosphatemia and to determine the effect of osteopontin and its functional fragments on such calcification, a relevant *in vitro* model system for calcification was developed, as follows.

Human vascular smooth muscle cells (HSMC) were obtained by enzymatic digestion as described by Ross, J. Cell Biol. 50:172-186 (1971) and Liaw et al., J. Clin. Invest. 95:713-724 (1995), incorporated herein by reference. Briefly, medial tissues were separated from segments of human aorta obtained at heart transplant surgery and autopsy, respectively. For plaque SMC, coronary atherectomy-derived tissues were obtained at time of surgery. Small pieces of tissue (1 to 2 mm^3) were digested overnight in DMEM supplemented with 165 U/ml collagenase type I, 15 U/ml elastase type III and 0.375 mg/ml soybean trypsin inhibitor at 37°C. Single cell suspensions were placed in 6-well plates and cultured for several weeks in DMEM supplemented with 20% FCS at 37°C in a humidified atmosphere containing 5% CO_2. Cultures that formed colonies were collected at confluence and maintained in growth medium (DMEM containing 15% FBS and 1 mM sodium pyruvate supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin; final inorganic phosphate concentration was 1.4 mM). Purity of cultures was assessed by positive immunostaining for alpha-SM actin and calponin and absence of von Willebrand factor staining as described by Ross, supra (1971) and Liaw et al., supra (1995).

Primary human adult and fetal aortic medial and coronary plaque primary cells up to passage 8 were used in these experiments. A fetal and adult HSMC culture was also immortalized using HPV-E6E7 and characterized as described by Perez et al., Proc. Natl. Acad. Sci. USA 89:1224-1228 (1992), incorporated herein by reference.

HSMC were routinely subcultured in growth medium. At confluence, cells were switched to calcification medium (DMEM containing 15% FBS and 1 mM

sodium pyruvate in the presence of 2 mM inorganic phosphate supplemented with 100 U/ml penicillin and 100 µg/ml of streptomycin) for up to 14 days. The medium was replaced with fresh medium every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0. Calcium deposition was quantified and assessed histochemically and cytochemically as described above in Example I.

In medium containing normal serum phosphate levels (inorganic phosphate, P_i, of 1.4 mM), HSMC accumulated very little calcium mineral. In contrast, in the presence of 2 mM inorganic phosphate, calcium deposition increased in a time-dependent manner. For example, on day 9, calcified HSMC vs. uncalcified control was 210.3 +/- 2.4 vs. 15.1 +/- 2.4 (µg/mg protein), mean +/- SEM (n+3)). The effect of inorganic phosphate on calcium deposition was dose-dependent over the range of 1.4 mM to 2 mM inorganic phosphate. Induction of calcification by elevated inorganic phosphate appeared to be a general feature of human cells, since primary HSMC derived from different sources (human adult and fetal aortic and coronary plaque) as well as immortalized derivatives of these cells showed similar behavior. No spontaneous deposition of calcium mineral occurred in calcification medium or in medium supplemented with up to 10 mM inorganic phosphate, indicating that cells and/or cell-derived matrix is necessary for mineralization.

To determine whether the observed calcification in the human cell culture system was physiologically relevant, morphological studies were performed. After culturing HSMC in calcification medium for 10 days, granular deposits developed throughout the cell culture. The deposits were identified as phosphate-containing

mineral by von Kossa staining, as described in Example I. Black-stained particles were diffusely scattered throughout the cell layer, predominantly in the extracellular regions, with greatest accumulation in multilayered foci. Electron microscopic analysis confirmed the presence of an apatite mineral phase, calcified collagen fibrils and matrix vesicles associated with mineralized cultures, essentially identical to the calcification of bovine SMC cultures in the system described in Example I.

These results demonstrate that HSMC cultures are susceptible to calcification when cultured in media containing inorganic phosphate concentrations typically found in hyperphosphatemic individuals. Furthermore, the observed calcification in the cultured human cells is similar to the ectopic calcification observed in calcified tissues *in vivo*. Therefore, the HSMC calcification culture system can be used to accurately assess the effect of regulators of ectopic calcification.

EXAMPLE IV

Inhibition of calcification of human vascular cells by osteopontin and functional fragments thereof

This example demonstrates that osteopontin and exemplary functional fragments of osteopontin effectively inhibit human smooth muscle cell calcification. Therefore, osteopontin can be used therapeutically to inhibit ectopic calcification.

Osteopontin proteins and functional fragments were assayed for their ability to inhibit ectopic calcification using the HSMC calcification system

described in Example III. The osteopontin proteins include full-length human recombinant osteopontin as well as recombinant N-terminal and C-terminal human osteopontin fragments similar to those that would be formed following thrombin cleavage of the native protein, as described by Smith et al., supra (1996). Two N-terminal fragments were used, 10N and 30N, which refer to two differ splice variants of osteopontin. The 30N splice variant contains an additional 14 amino acids, NAVSSEETNDFKQE (SEQ ID NO:7), which correspond to exon 5 (amino acids 59-72). The 10N fragment contains amino acids 17-58 and 73-160 of native osteopontin, whereas the 30N fragment contains amino acids 17-169. The 10C fragment contains the C-terminal domain of osteopontin, amino acids 170-317.

The N- and C-terminal recombinant osteopontin fragments were expressed as fusion proteins with GST, purified from bacterial lystates by affinity chromatography on glutathione beads, and cleaved with thrombin. The full-length human recombinant osteopontin was prepared as a His-tagged protein. The size and purity of the resulting recombinant proteins was confirmed by SDS-PAGE analysis (Smith et al., supra (1996)).

Recombinant osteopontin and its functional fragments were assayed for their ability to inhibit ectopic calcification of human smooth muscle cells (HSMC) either prior to or following phosphorylation by casein kinase II. The amount of phosphate incorporated into osteopontin (OPN) and its fragments achieved by casein kinase II phosphorylation is shown in Figure 5b. As shown in Figure 5a, in the presence of high-phosphate calcification medium, calcium deposition into HSMC matrix

is reduced to basal levels by the addition of phosphorylated OPN, 30N OPN, 10N OPN or 10C OPN. The non-phosphorylated forms of these proteins do not significantly affect calcium deposition in this assay. These results show that both N- and C-terminal fragments of osteopontin are functional fragments of osteopontin, and that serine-threonine phosphorylation appears to be important for the functional activity of osteopontin and its functional fragments.

As shown in Figure 6, recombinant osteopontin phosphorylated by casein kinase II is able to inhibit HSMC calcification at a concentration of 15 nM. Dephosphorylation with alkaline phosphatase (ALP) reverses this inhibitory ability. These results confirm the importance of phosphorylation for the functional activity of osteopontin and its functional fragments.

The effect of human osteopontin on inhibiting ectopic calcification is dose-dependent over the range of concentrations of 0.1 µg/ml to 5.0 µg/ml (Figure 7). Furthermore, the effect of osteopontin on inhibiting and reversing ectopic calcification is rapid, with significantly reduced calcium deposition being apparent by 60 minutes, with approximately 50% inhibition observable by 90 minutes following addition (Figure 8).

These results indicate that osteopontin and exemplary functional fragments thereof are able to effectively inhibit physiologically relevant ectopic calcification of human cells rapidly and at low concentrations. Therefore, full-length osteopontin and functional fragments thereof will be therapeutically

effective in inhibiting ectopic calcification in individuals exhibiting or at risk of exhibiting ectopic calcification.

EXAMPLE IV

Osteopontin inhibits ectopic calcification *in vivo*

This example shows that osteopontin inhibits ectopic calcification *in vivo*.

The effect of subcutaneous implantation of porcine prosthetic valves in normal mice and mice deficient in osteopontin was tested to determine the role of osteopontin in ectopic calcification *in vivo*. Mice deficient in one or both copies of the osteopontin gene are described in Liaw et al., supra (1998). A 4.0 mm^2 piece of porcine glutaraldehyde-fixed aortic valve leaflet was subcutaneously implanted into 5-6 week old female mice carrying either the wild type (WT), heterozygote (HTZ) or null allele (KO) for osteopontin. After 14 days, implants were removed, freeze-dried and acid hydrolyzed. Calcium levels were assayed as described in Example I and normalized to the dried weight of the explant.

As shown in Figure 9, implanted valves calcify to a significantly greater extent in osteopontin null mice than in wild-type or heterozygous mice. Therefore, consistent with the observed ability of osteopontin to inhibit ectopic calcification in relevant *in vitro* systems, these results indicate that osteopontin inhibits ectopic calcification *in vivo*.

The foreign body inflammatory response also appears to be impaired in the osteopontin null mouse. For example, there is an apparent reduction in infiltration by macrophages at the site of valve implantation in the osteopontin null mouse compared to the wild-type or heterozygous mice. Macrophages that normally infiltrate a site of inflammation and ectopic calcification are contemplated to promote removal of calcified deposits by phagocytosis. Therefore, it is contemplated that osteopontin both inhibits hydroxyapatite formation and promotes phagocytotic resorption of calcified deposits by macrophages.

Accordingly, the administration of osteopontin or its functional fragments to an individual will be therapeutically effective in inhibiting ectopic calcification.

Example V

Osteopontin-mediated inhibition of ectopic calcification *in vivo* by CAII expressing foreign body giant cells (FBGC) and macrophages

This example demonstrates that osteopontin promotes the accumulation and activation of carbonic anhydrase II expressing macrophages and osteoclast-like foreign body giant cells (FBGC) capable of acidifying the extracellular milieu and dissolving mineralized deposits.

Implantation of Aortic Valve Leaflets into OPN-Deficient Mice

The effect of subcutaneous implantation of a bioprosthetic glutaraldehyde-fixed aortic valve (GFAV) in normal mice and mice deficient in osteopontin was tested to further characterize the role of osteopontin in ectopic calcification *in vivo*. As described in Example V, mice deficient in one or both copies of the osteopontin gene were obtained according to Liaw et al., supra (1998). A 4.0 mm^2 piece of glutaraldehyde-fixed aortic valve leaflet (GFAV) was subcutaneously implanted into 5-6 week old female mice carrying either the wild type (WT), heterozygote (HTZ) or null allele (KO) for osteopontin. After 7, 14, and 30 days, implants were removed from osteopontin wild type (WT), heterozygote (HTZ) or null allele (KO) mice and assayed for mineral deposition, protein accumulation, and cell recruitment.

Once removed, the implants were fixed with methyl carnoys solution, embedded in paraffin, and 5μm sections were analyzed for OPN accumulation using an anti-OPN (OP-199) antibody at 10 μg/ml as described by Liaw et al., *supra*. Sections were counter stained with methyl green. OPN accumulation was quantitated using the Pro Image Analysis Program. In addition, calcium levels were assayed as described in Example I and normalized to the dried weight of the implant.

As shown in Figure 10A, osteopontin is observed within valves after 14 days of implantation into osteopontin WT mice. At this time, osteopontin is restricted to the border between the implant and the surrounding foreign body response. In contrast, the

osteopontin HTZ and KO mice have greatly reduced or absent OPN levels at 14 days of GFAV implantation, respectively.

To quantitate GFAV calcification, the explants were freeze dried, weighed, then acid hydrolyzed with 0.6 N HCL overnight at room temperature. Calcium quantitation was performed by the o-cresolphtalein complexone as directed in the Sigma Diagnostic kit (Sigma, St. Louis, IL) and normalized to dry weight. Accuracy of the kit was confirmed by atomic absorption spectroscopy. The osteopontin WT mice show no detectable GFAV mineralization based on Alizarin Red S staining and calcium quantitation. In contrast, the osteopontin HTZ and KO mice show GFAV calcification 4-to 5-fold higher than the osteopontin WT mice (Figure 10B).

Within 30 days, the osteopontin KO mice still do not accumulate osteopontin and GFAV mineralization is not significantly changed from the 14 day time point (Figures 10A and 10B). In the osteopontin HTZ and WT mice show elevated osteopontin levels with respect to the 14 day time point. In these mice, osteopontin localization is no longer restricted to the border of the implant/foreign body capsule, but is also found to penetrate the GFAV implant. In addition, the osteopontin localized to the foreign body capsule is most highly expressed by the FBGCs adjacent to the implant. At the 30 day time point, osteopontin WT mice exhibit GFAV calcification, but mineral levels are 4-fold lower than observed for osteopontin HTZ and KO mice. Also at the 30 day time point, osteopontin HTZ mice demonstrate a dramatic reduction in calcification, demonstrating that the presence of osteopontin is not only inhibiting mineral deposition, but also mediating the removal of

mineral from the GFAV. At the 60 day time point, osteopontin HTZ and WT mice exhibit a 4-5 fold increase compared to the respective 30 day time point (Figure 10B). Similarly, at the 60 day timepoint, GFAV valve calcification in osteopontin KO mice increases almost 2-fold compared to the 30 day time point (Figure 10B).

The accumulation of significant osteopontin levels observed at the 30 day timepoint in osteopontin HTZ mice was concurrent with a reduction or reversal in GFAV mineralization. Since OPN alone cannot mediate the dissolution of calcium phosphate, the observed mineral loss is rather an osteopontin-regulated event. The only mechanisms capable of calcium phosphate removal are phagocytosis and acidification.

Analysis of Osteopontin-Mediated Macrophage Recruitment

To examine macrophage accumulation GFAV implants removed from osteopontin WT, HTZ and KO mice at 14 days and 30 days and 5 µm sections were analyzed for macrophage accumulation using rat anti-mouse BM-8 (Accurate Chemical & Scientific Corp., Westbury, N.Y.) at 6 µg/ml. Following a 1 hour incubation with primary antibody, biotinylated goat anti-rat antibody (Vector Laboratories Inc., Burlingame, CA) was added and after 45 minutes the reaction product was detected with 3,3'-diaminobenzidine (DAB)(Sigma, St. Louis, MO). The sections were counterstained with methyl green

As represented by the immunochemical localization and quantitation of the BM-8 surface marker, the osteopontin KO mice display a defect in macrophage recruitment to the GFAV implantation site (Figure 11).

In addition, the osteopontin KO mice demonstrated BM-8 cell staining at levels 50%-25% lower compared to the osteopontin WT mice at 14 and 30 days of implantation, respectively.

In osteopontin WT mice, BM-8 positive cells accumulated diffusively at early time points, but later became concentrated along the edge of the implant suggesting their activation. In contrast, the BM-8 cells failed to become concentrated along the implant/foreign body capsule border in the osteopontin KO mice implying an activation defect.

While osteopontin HTZ mice also displayed decreased BM-8 positive cell accumulation, the macrophage defect did not correlate with the mineral loss pattern observed at the 30 day time point. Overall, very few macrophages were able to penetrate the GFAV at any time point examined, suggesting that phagocytosis alone cannot mediate mineralization reversal.

GFAV Explant pH Analysis

GFAV were explanted from osteopontin WT, HTZ, and KO mice after 30 days of implantation to investigate whether osteopontin promotes implant acidification, contributing to the removal of mineral from the GFAV implant as observed in osteopontin HTZ mice. The explants were freeze-dried and then immersed in Universal Indicator Solution (Fisher Scientific, Pittsburgh, PA). The pH of each implant was independently determined by three different observers comparing the color of the GFAV solution to the manufacturer's provided color scale.

While GFAV explants from the osteopontin WT mice had an acidic pH (6.0), explants from osteopontin KO mice maintained a near neutral pH (6.7). These differences in pH were statistically significant ($p=.0002$). GFAV implants from osteopontin HTZ mice had an intermediate pH (6.2). Unimplanted GFAV had a pH of 6.5 (Figure 12). Since apatite stability decreases at pHs less than 7.0 at 37° C, the observed pHs of the explants would be physiologically relevant with the respect to the ability to dissolve apatitic mineral deposits and explain the observed loss in GFAV mineralization. These findings demonstrate that osteopontin controls mineral resorption by regulating the ability of host cells to acidify the GFAV microenvironment.

Analysis of Carbonic Anhydrase II Expression at the GFAV Implantation Site

Osteoclasts and macrophages express carbonic anhydrase II (CAII), an enzyme that promotes acidification of the local microenvironment. CAII and cathepsin K expressing cells were localized in GFAV explants from osteopontin WT, HTZ, and KO mice at 14 days and 30 days after implantation by localizing protein in 5 µm sections using a sheep anti-human CAII antibody (Biodesign International, Kennebunkport, ME) at 5µg/ml. Incubation with primary antibody at room temperature for 1 hour was followed by incubation with a biotinylated rabbit anti-sheep antibody (Vector Laboratories Inc., Burlingame, CA) for 45 minutes. The reaction product was detected with 3,3'-diaminobenzidine (DAB)(Sigma, St. Louis, MO) and sections were counterstained with methyl green. CAII expression was quantitated using the Pro Image Analysis Program and is presented as percent area

in Figure 13. FBGC were counted in 4 quadrants of the explant (Figure 14). Any cell containing more than one nucleus was considered a FBGC.

Quantitation of CAII immunostaining revealed a mineral- and osteopontin dependent regulation of CAII expression (Figure 13). At 14 days, osteopontin is present and GFAV implants do not mineralize in osteopontin WT mice. Subsequently, when there is little requirement for mineral removal, CAII levels are low. At 30 days GFAV begin to mineralize, osteopontin accumulates, and CAII expressing cells are dramatically increased. Conversely, the osteopontin KO mice, which display elevated mineral levels at all observed time points, show very low CAII levels. The osteopontin mediated CAII response is accelerated in the osteopontin HTZ mice. At 14 days, osteopontin HTZ mice demonstrate substantial GFAV mineralization due to depressed levels of osteopontin, but respond to the mineralization by producing CAII-positive cells. Thus, the data demonstrate that osteopontin HTZ mice do not express high enough levels osteopontin to directly inhibit mineralization, but the expression level is sufficient to promote CAII expression or promotion of CAII-positive cell formation. In addition to CAII, high expression of cathepsin K, a cysteine protease expressed predominantly in osteoclasts and shown to be critical for osteoclast resorption activity, was observed in CAII-expressing FBGC.

To determine whether the acidification deficiency observed in osteopontin HTZ and osteopontin KO mice was a consequence of a reduced number of FBGCs, the number of FBGCs on GFAV explants was counted. FBGCs were visualized by electron microscopy performed as described

by Wada et al., Cir. Res. 84, 166-178 (1999), which is incorporated herein by reference. The number of FBGCs is enhanced 2-3 fold in OPN-deficient, highly calcified conditions represented by 14 day GFAV explants from osteopontin HTZ and KO mice.

The pattern of FBGC accumulation is almost identical to the calcium deposition pattern, and inverse to OPN accumulation. This data suggests that osteopontin deficiency promotes an increase in FBGC accumulation near the GFAV implant, but these FBGC demonstrate reduced acidicfication potential as measured by decreased CAII levels.

These results indicate that osteopontin promotes the accumulation and activation of carbonic anhydrase II expressing macrophages and osteoclast-like foreign body giant cells (FBGC) capable of acidifying the extracellular milieu and dissolving mineralized deposits.

Phosphorylated Osteopontin Inhibits Bioprosthetic Valve Calcification *In Vivo*

GFAV were incubated in a 1 mg/ml solution of either control vehicle, unphosphorylated osteopontin or phosphorylated osteopontin for three days prior to implantation into osteopontin KO mice. After 14 days calcium quantitation was performed as described above. As shown in Figure 15, these results indicate that phosphorylated osteopontin inhibits bioprosthetic valve calcification *in vivo*.

What is claimed is:

1. A method of inhibiting ectopic calcification in an individual, comprising administering to said individual a therapeutically effective amount of osteopontin or a functional fragment thereof.

2. The method of claim 1, wherein said ectopic calcification is associated with a condition selected from the group consisting of atherosclerosis, stenosis, restenosis, prosthetic valve replacement, angioplasty, renal failure, tissue injury, diabetes and aging.

3. The method of claim 1, wherein said osteopontin is a polypeptide comprising substantially the amino acid sequence of SEQ ID NO:2, or a functional fragment thereof.

4. The method of claim 1, wherein said osteopontin or functional fragment thereof is administered with a pharmaceutically acceptable carrier.

5. The method of claim 1, wherein said osteopontin or functional fragment thereof is administered at the site of ectopic calcification.

6. The method of claim 5, wherein said osteopontin or functional fragment thereof contacts a prosthetic device.

7. The method of claim 6, wherein said prosthetic device is a bioprosthetic heart valve.

8. The method of claim 6, wherein said contacting comprises attachment of osteopontin or a functional fragment thereof to said prosthetic device.

9. The method of claim 6, wherein said contacting comprises attachment of cells producing said osteopontin or a functional fragment thereof to said prosthetic device.

10. The method of claim 9, wherein said cells recombinantly produce osteopontin or a functional fragment thereof.

11. The method of claim 1, wherein said osteopontin interacts with hydroxyapatite crystals.

12. The method of claim 1, wherein said osteopontin stimulates cells to acidify the local microenvironment.

13. The method of claim 12, wherein said cells express carbonic anhydrase II.

14. The method of claim 13, wherein said cells are multinucleate foreign body giant cells.

15. The method of claim 13, wherein said cells are macrophages.

16. A method of treating or inhibiting ectopic calcification comprising administering to an individual macrophages, whereby said macrophages are targeted to the site of ectopic calcification.

17. The method of claim 16, wherein said macrophages acidify the local microenvironment by expressing carbonic anhydrase II.

18. The method of claim 16, further comprising administering osteopontin, thereby stimulating said acidification of the local microenvironment by said macrophages.

19. The method of claim 16, wherein said macrophages contact a prosthetic device.

20. The method of claim 19, wherein said prosthetic device is a bioprosthetic heart valve.

21. A method of treating or inhibiting ectopic calcification comprising administering to an individual multinucleate foreign body giant cells, whereby said multinucleate foreign body giant cells are targeted to the site of ectopic calcification.

22. The method of claim 21, wherein said multinucleate foreign body giant cells acidify the local microenvironment by expressing carbonic anhydrase II.

23. The method of claim 21, further comprising administering osteopontin, thereby stimulating said acidification of the local microenvironment by said multinucleate foreign body giant cells.

24. The method of claim 21, wherein said multinucleate foreign body giant cells contact a prosthetic device.

25. The method of claim 24, wherein said prosthetic device is a bioprosthetic heart valve.

26. A method of treating or inhibiting ectopic calcification in an individual comprising promoting recruitement of acid producing cells to a site of ectopic calcification by administering osteopontin.

27. The method of claim 26, wherein said acid producing cells express carbonic anhydrase II.

28. The method of claim 26, wherein said acid producing cells are macrophages.

29. The method of claim 26, wherein said acid producing cells are multinucleate foreign body giant cells.

30. A method of treating or inhibiting ectopic calcification in an individual comprising increasing expression of carbonic anhydrase II at a site of ectopic calcification by administering osteopontin.

31. The method of claim 30, wherein carbonic anhydrase II is expressed by multinucleate foreign body giant cells.

32. The method of claim 30, wherein carbonic anhydrase II is expressed by macrophages.

```
   1  GACCAGACTC GTCTCAGGCC AGTTGCAGCC TTCTCAGCCA AACGCCGACC AAGGAAAACT CACTACCATG AGAATTGCAG TGATTTGCTT TTGCCTCCTA
 101  GGCATCACCT GTGCCATACC AGTTAAACAG GCTGATTCTG GAAGTTCTGA GGAAAAGCAG CTTTACAACA AATACCCAGA TGCTGTGGCC ACATGGCTAA
 201  ACCCTGACCC ATCTCAGAAG CAGAATCTCC TAGCCCCACA GAATGCTGTG TCCTCTGAAG AAACCAATGA CTTTAAACAA GAGACCCTTC CAAGTAAGTC
 301  CAACGAAAGC CATGACCACA TGGATGATAT GGATGATGAA GATGATGATG ACCATGTGGA CAGCCAGGAC TCCATTGACT CGAACGACTC TGATGATGTA
 401  GATGACACTG ATGATTCTCA CCAGTCTGAT GAGTCTCACC ATTCTGATGA ATCTGATGAA CTGGTCACTG ATTTTCCCAC GGACCTGCCA GCAACCGAAG
 501  TTTTCACTCC AGTTGTCCCC ACAGTAGACA CATATGATGG CCGAGGTGAT AGTGTGGTTT ATGGACTGAG GTCAAAATCT AAGAAGTTTC GCAGACCTGA
 601  CATCCAGTAC CCTGATGCTA CAGACGAGGA CATCACCTCA CACATGGAAA GCGAGGAGTT GAATGGTGCA TACAAGGCCA TCCCCGTTGC CCAGGACCTG
 701  AACGCGCCTT CTGATTGGGA CAGCCGTGGG AAGGACAGTT ATGAAACGAG TCAGCTGGAT GACCAGAGTG CTGAAACCCA CAGCCACAAG CAGTCCAGAT
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 901  TCACAGCCAT GAAGATATGC TGGTTGTAGA CCCCAAAAGT AAGGAAGAAG ATAAACACCT GAAATTTCGT ATTTCTCATG AATTAGATAG TGCATCTTCT
1001  GAGGTCAATT AAAAGGAGAA AAAATACAAT TTCTCACTTT GCATTTAGTC AAAAGAAAAA ATGCTTTATA GCAAAATGAA AGAGAACATG AAATGCTTCT
1101  TTCTCAGTTT ATTGGTTGAA TGTGTATCTA TTTGAGTCTG GAAATAACTA ATGTGTTTGA TAATTAGTTT AGTTTGTGGC TTCATGGAAA CTCCCTGTAA
1201  ACTAAAAGCT TCAGGGTTAT GTCTATGTTC ATTCTATAGA AGAAATGCAA ACTATCACTG TATTTTAATA TTTGTTATTC TCTCATGAAT AGAAATTTAT
1301  GTAGAAGCAA ACAAAATACT TTTACCCACT TAAAAAGAGA ATATAACATT TTATGTCACT ATAATCTTTT GTTTTTTAAG TTAGTGTATA TTTTGTTGTG
1401  ATTATCTTTT TGTGGTGTGA ATAA
```

Fig. 1A

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HUMAN  1  MRIAVICFCLLGITCAIPVK@ADSGSSEEK@LYNKYPDAVATWLNPDPSQK@NLLAP@NAVSSEETNDFK@ETLPSKSNESHDHMDDMDDEDDD-DHVDS
     101  @DSIDSNDSDDVDDTDDSH@SDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRPDI@YPDATDEDITSHMESEELN
     201  GAYKAIPVA@DLNAPSDWDSRGKDSYETS@LDD@SAETHSHK@SRLYKRKANDESNEHSDV----------IDS@ELSKVSREFHSHEFHSH
     301  EDMLVVDPKSKEEDKHLKFRISHELDSASSEVN
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Fig. 1B

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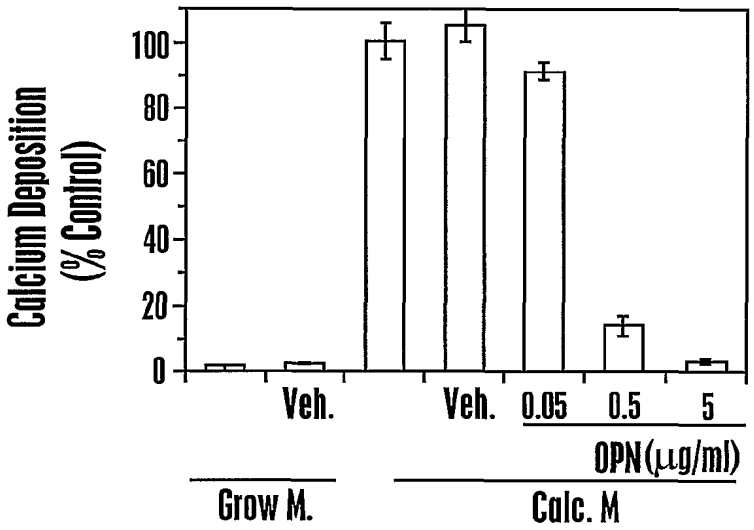

Calcium Deposition
(% Control)
100
80
60
40
20
0
Veh.
Veh.
0.05
0.5
5
OPN(μg/ml)
Grow M.
Calc. M

Fig. 2A

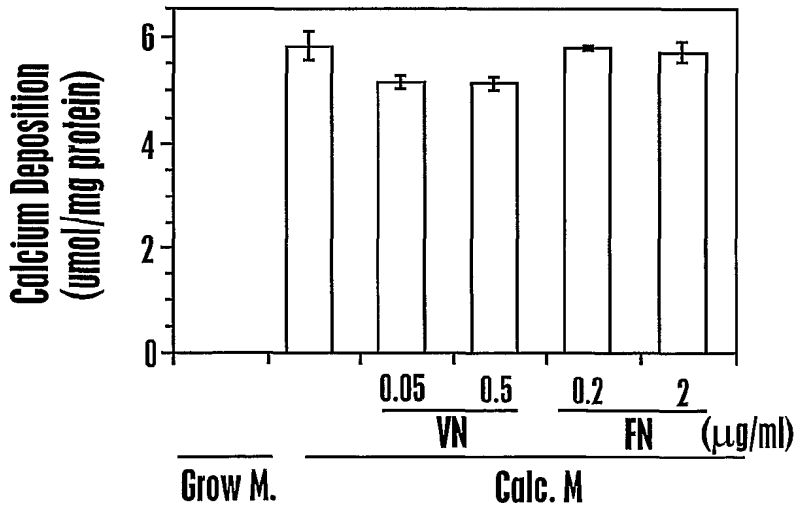

Calcium Deposition
(umol/mg protein)
6
4
2
0
0.05
0.5
0.2
2
VN
FN
(μg/ml)
Grow M.
Calc. M

Fig. 2B

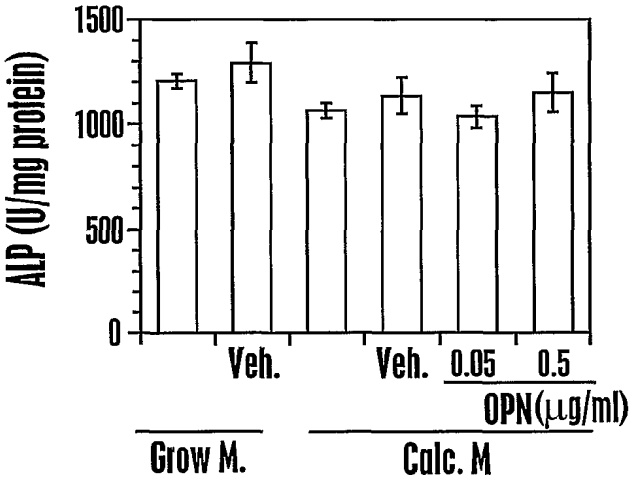


Fig. 3A

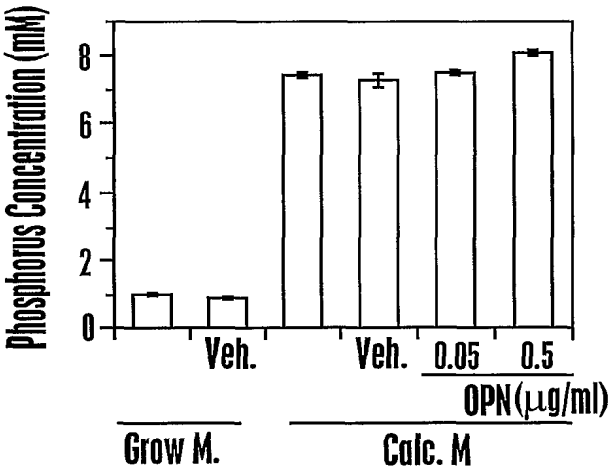


Fig. 3B

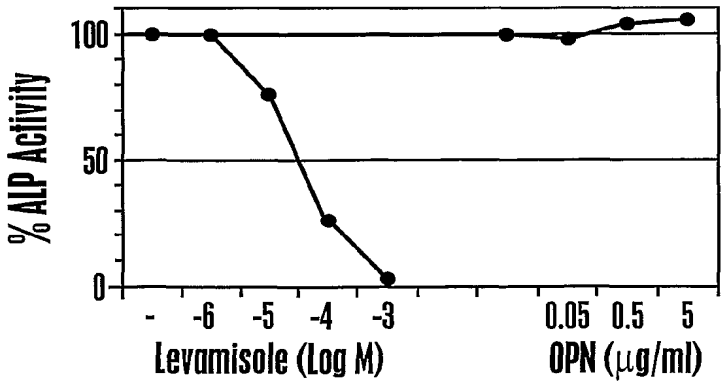


Fig. 3C

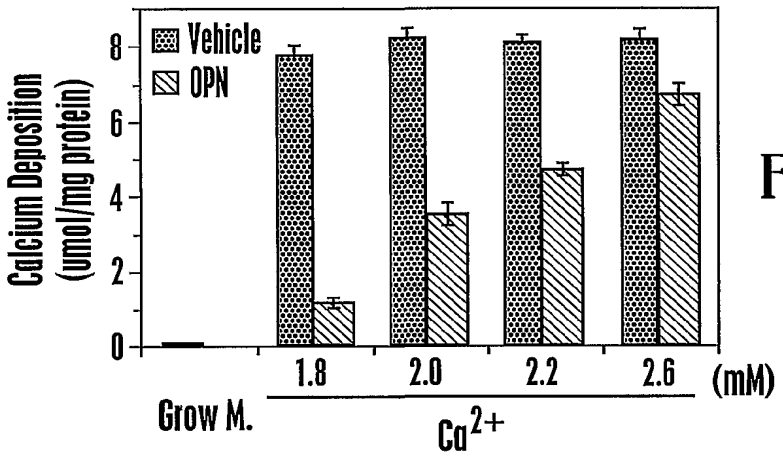


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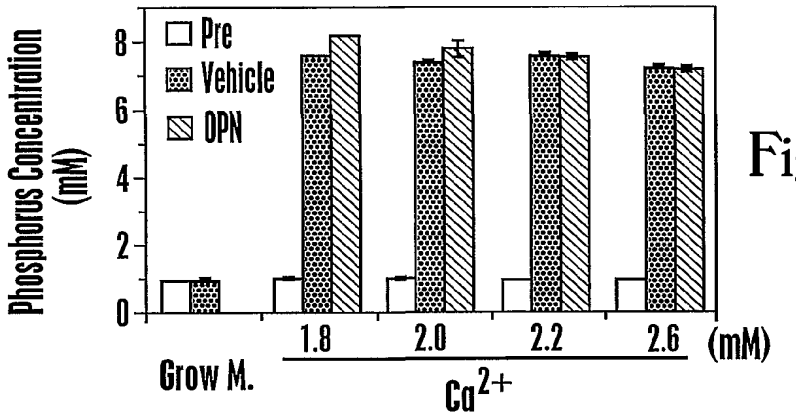


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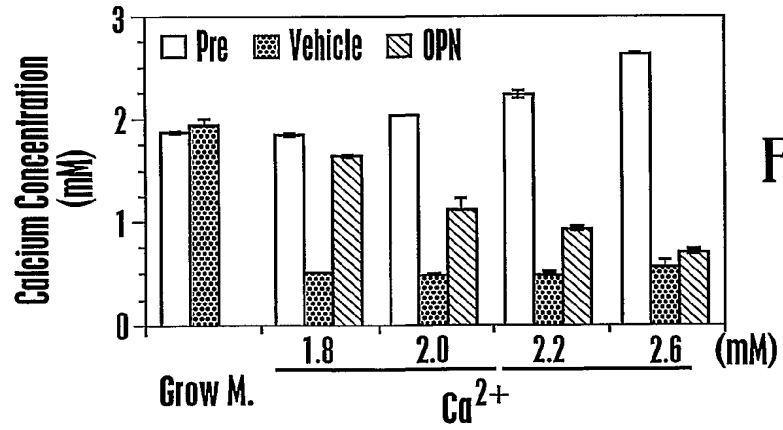


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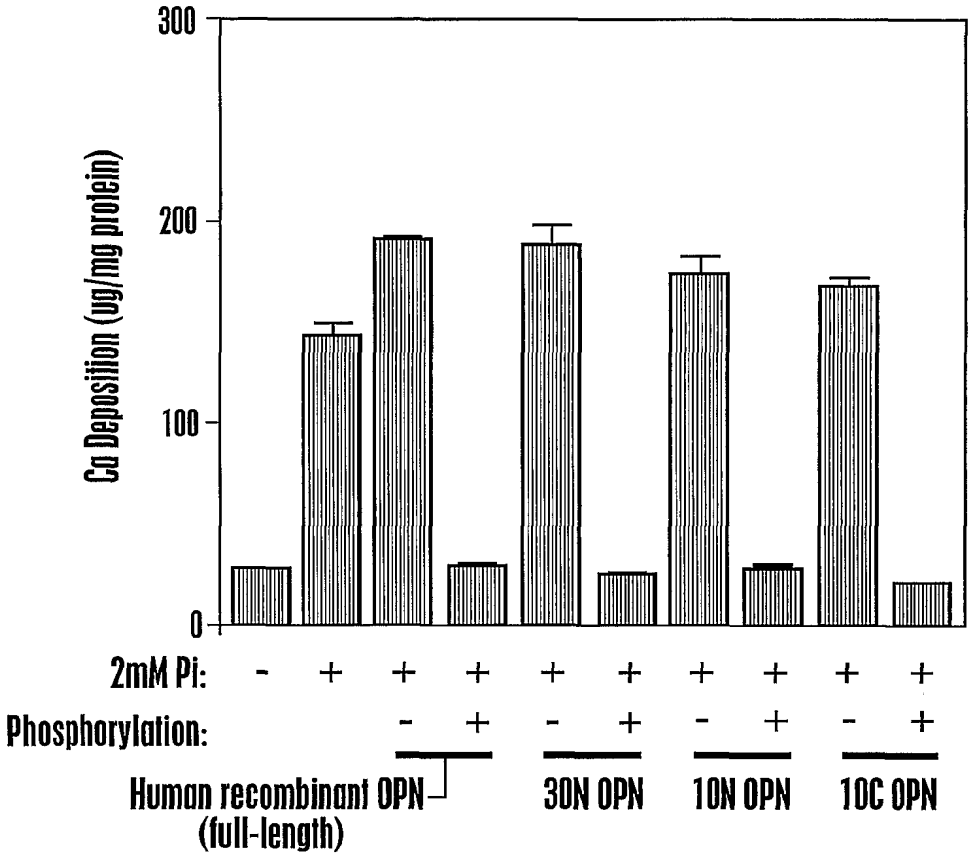


Fig. 5A

OPN	mol of phosphate/mol of OPN
Full-length	20
30N	12
10N	8.9
10C	8.9

Fig. 5B

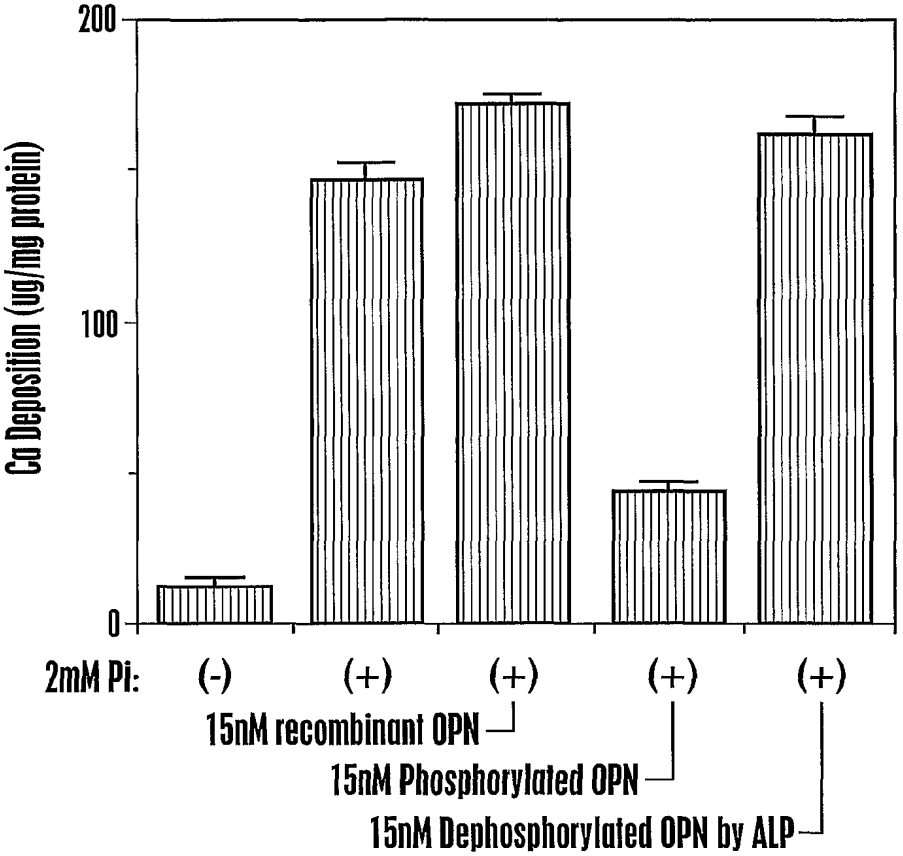

200
100
0
Ca Deposition (ug/mg protein)
2mM Pi: (-) (+) (+) (+) (+)
15nM recombinant OPN
15nM Phosphorylated OPN
15nM Dephosphorylated OPN by ALP

Fig. 6

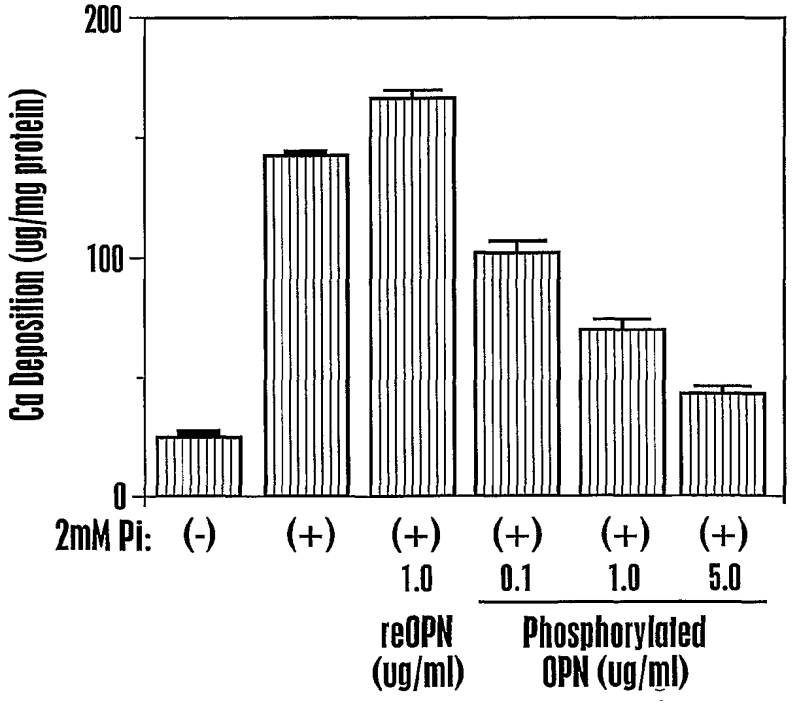

200
100
0
Ca Deposition (ug/mg protein)
2mM Pi: (-) (+) (+) (+) (+) (+)
1.0 0.1 1.0 5.0
reOPN (ug/ml)
Phosphorylated OPN (ug/ml)

Fig. 7

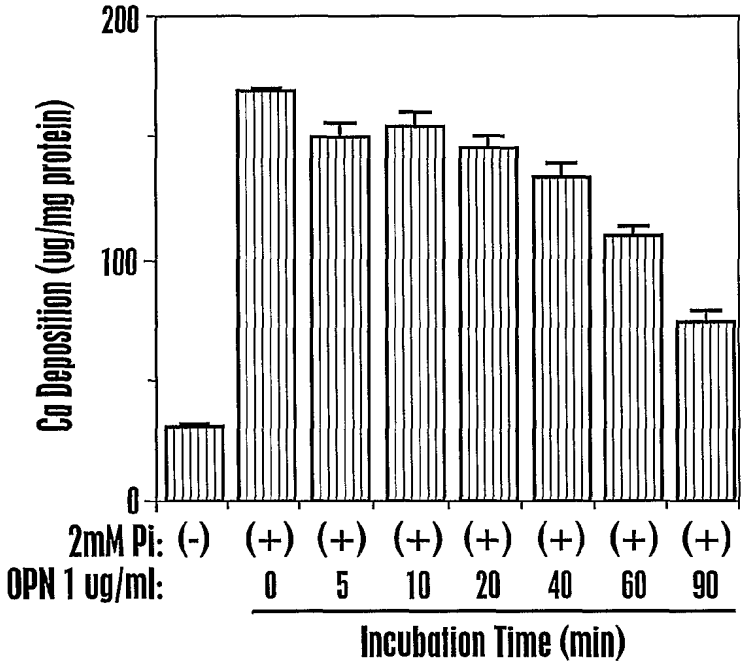

200
100
0
Ca Deposition (ug/mg protein)
2mM Pi: (-) (+) (+) (+) (+) (+) (+) (+)
OPN 1 ug/ml: 0 5 10 20 40 60 90
Incubation Time (min)

Fig. 8

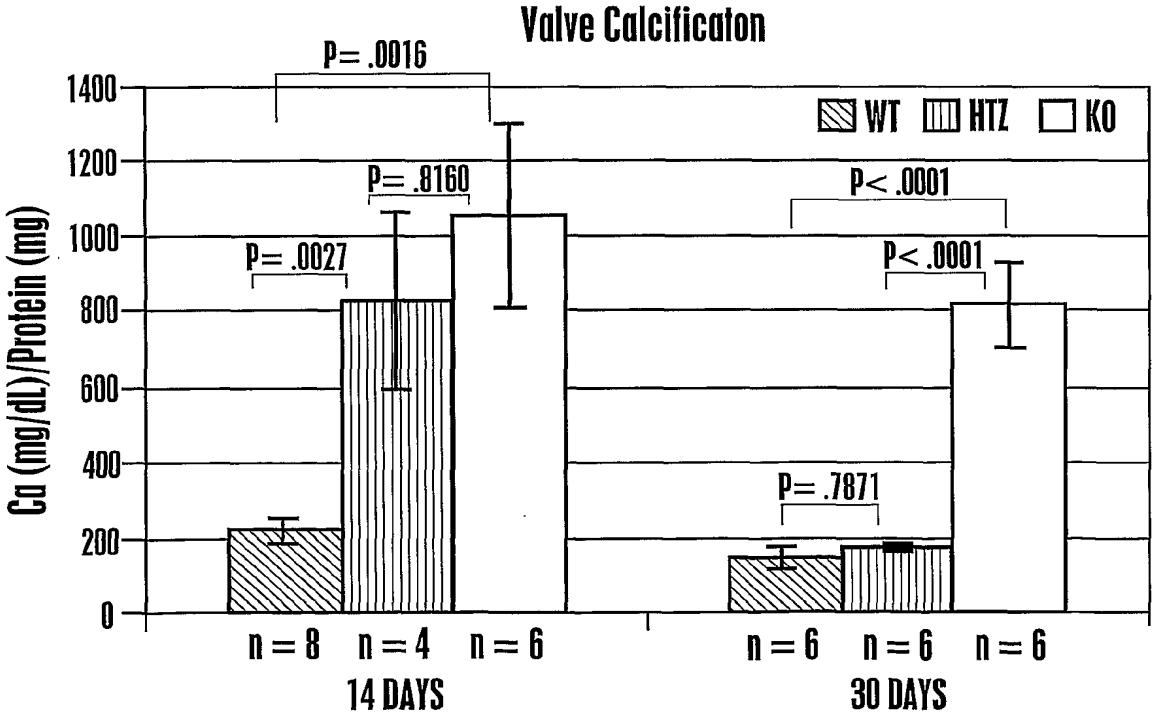

Valve Calcificaton
P= .0016
P= .8160
P= .0027
P< .0001
P< .0001
P= .7871
WT HTZ KO
1400
1200
1000
800
600
400
200
0
Ca (mg/dL)/Protein (mg)
n = 8 n = 4 n = 6
14 DAYS
n = 6 n = 6 n = 6
30 DAYS

Fig. 9

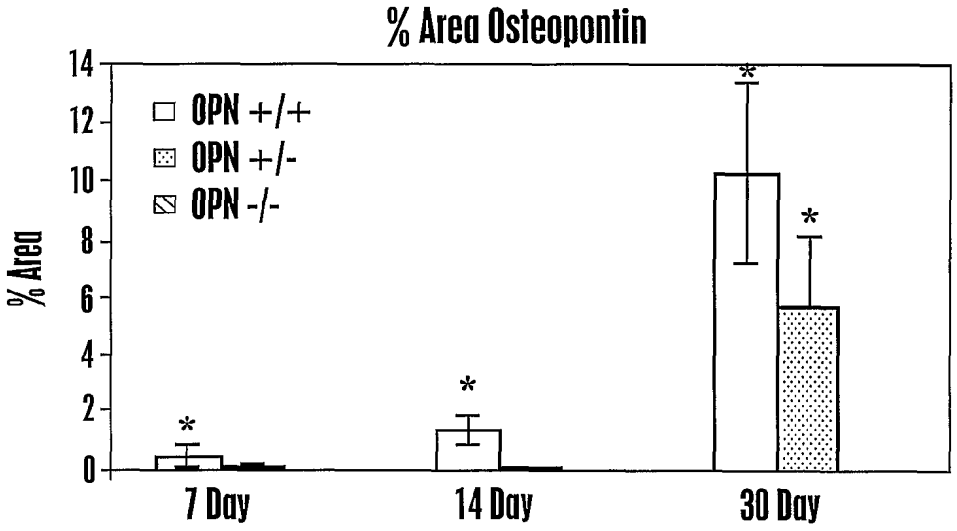


Fig. 10A

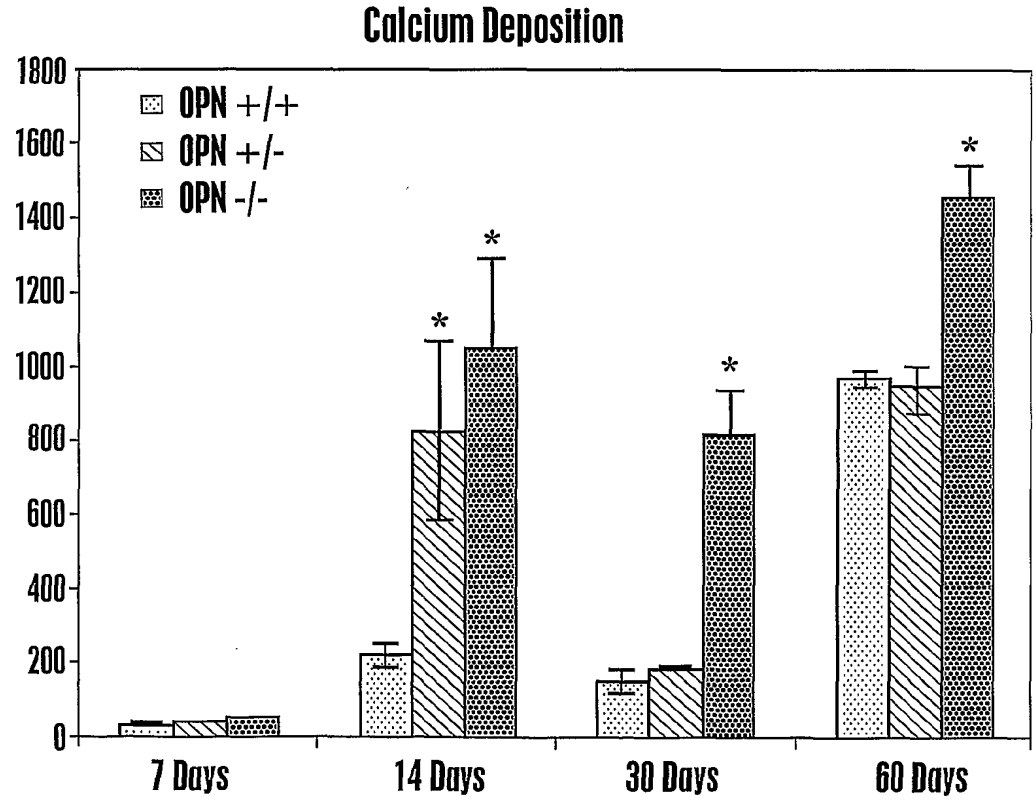


Fig. 10B

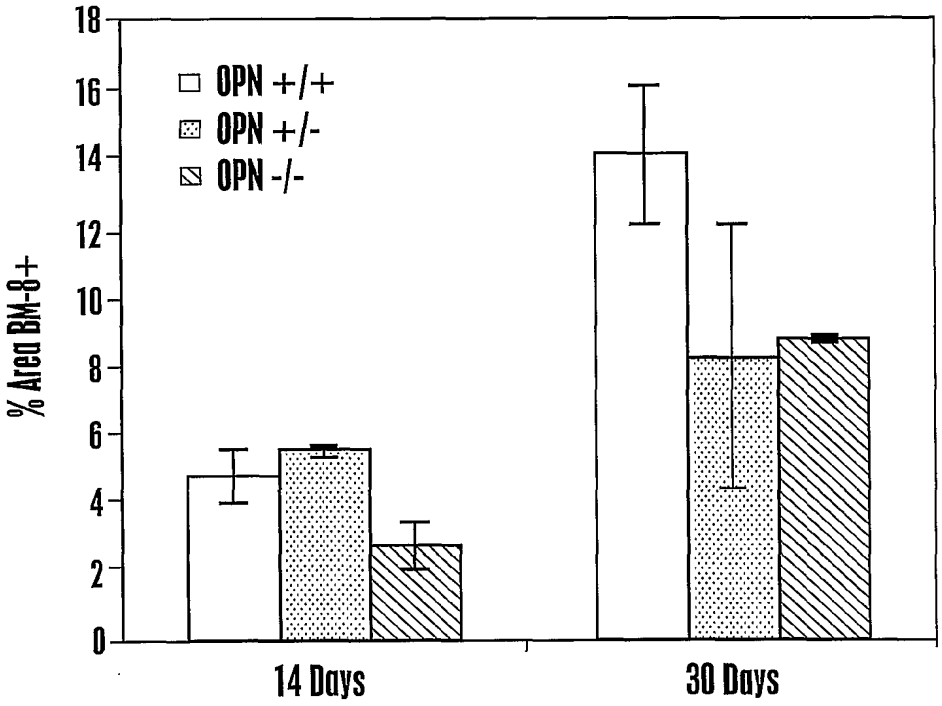

18
16
14
12
10
8
6
4
2
0
% Area BM-8+
OPN +/+
OPN +/-
OPN -/-
14 Days
30 Days

Fig. 11

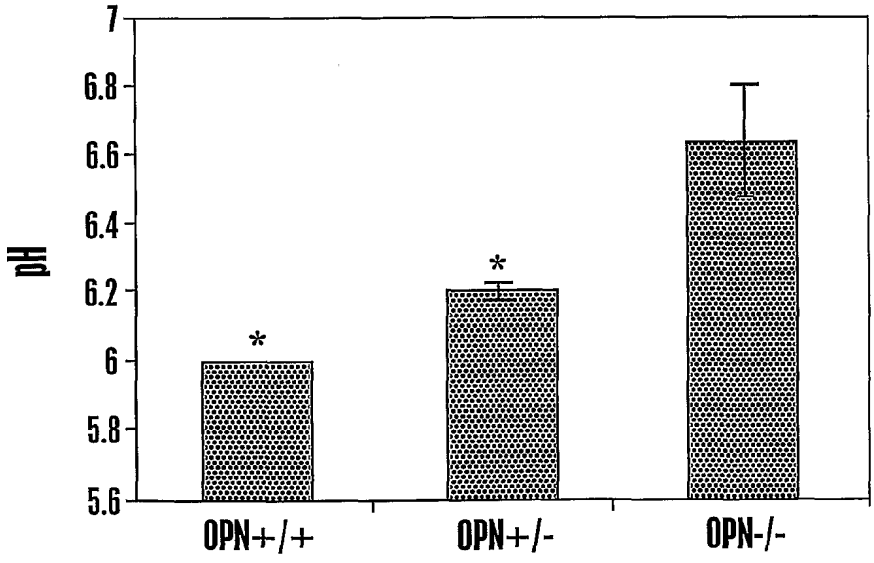

7
6.8
6.6
6.4
6.2
6
5.8
5.6
pH
*
*
OPN+/+
OPN+/-
OPN-/-

Fig. 12

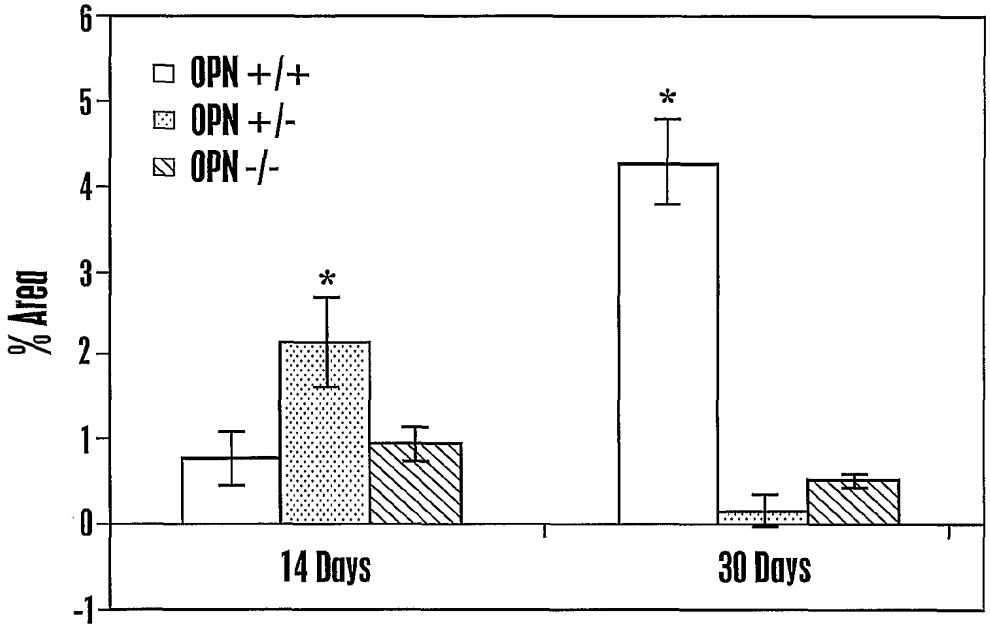

% Area
6
5
4
3
2
1
0
-1
OPN +/+
OPN +/-
OPN -/-
*
*
14 Days
30 Days

Fig. 13

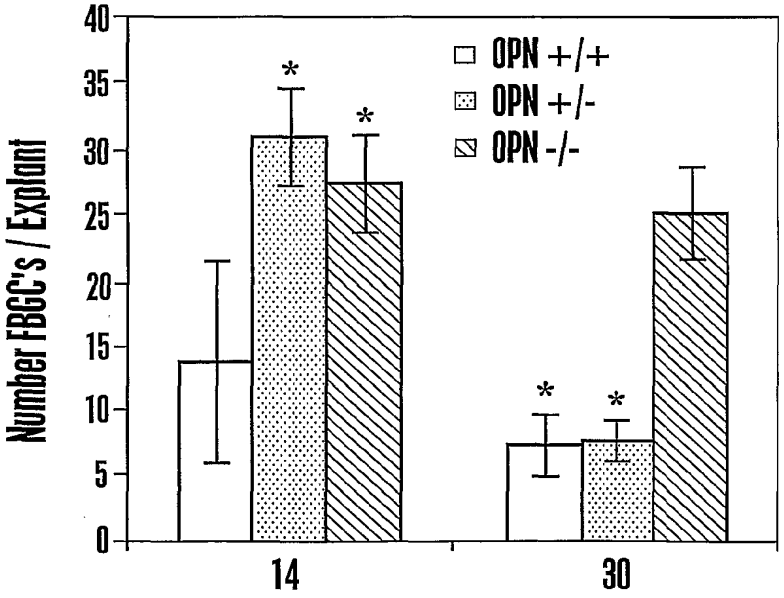

Number FBGC's / Explant
40
35
30
25
20
15
10
5
0
OPN +/+
OPN +/-
OPN -/-
*
*
*
*
14
30

Fig. 14

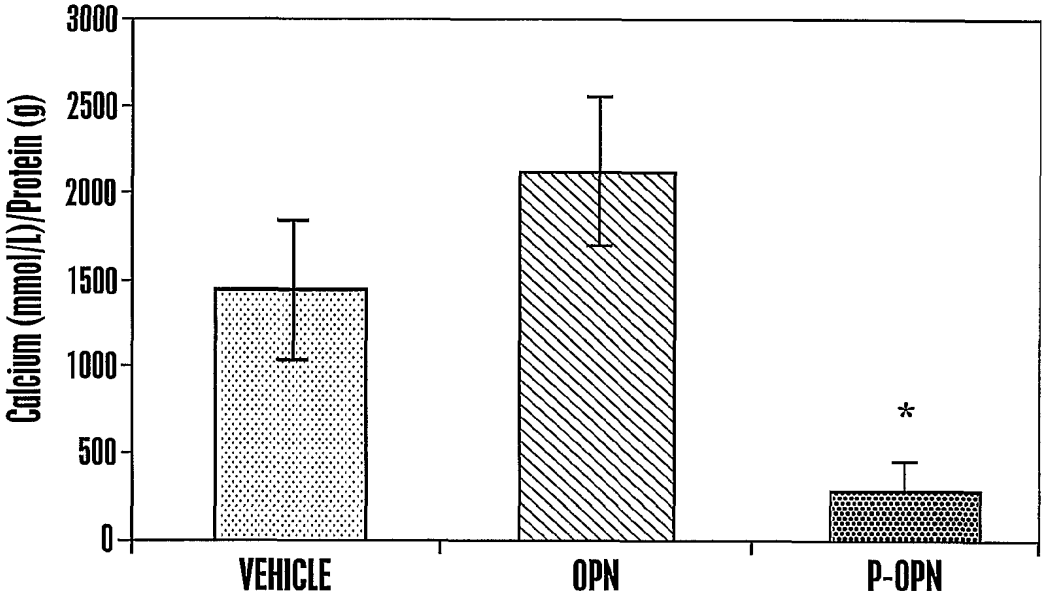

3000
2500
2000
1500
1000
500
0
Calcium (mmol/L)/Protein (g)
*
VEHICLE
OPN
P-OPN

Fig. 15

SEQUENCE LISTING

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Steitz, Susie
University of Washington

<120> Methods of Inhibiting Ectopic Calcification

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 95                 100                 105                 110

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Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser
                115                 120                 125

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Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala
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acc gaa gtt ttc act cca gtt gtc ccc aca gta gac aca tat gat ggc   541
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cga ggt gat agt gtg gtt tat gga ctg agg tca aaa tct aag aag ttt   589
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cgc aga cct gac atc cag tac cct gat gct aca gac gag gac atc acc   637
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175                 180                 185                 190

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                275                 280                 285

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Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile
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        305                 310

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Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His
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Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys Asp Ser
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225                 230                 235                 240

Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu
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INTERNATIONAL SEARCH REPORT

Int[illegible]ional Application No
PCT/US 99/29173

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/19 A61K35/14 A61P9/10 A61P13/12 A61P3/00
A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIACHELLI, CECILIA M. ET AL: "Osteopontin: Potential roles in vascular function and dystrophic calcification" J. BONE MINER. METAB. (1997), 15(4), 179-183 , XP000886502	1-15, 26-32
Y	page 181, column 2 ---	16-20
Y	O'BRIEN E R ET AL: "Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques." ARTERIOSCLEROSIS AND THROMBOSIS, (1994 OCT) 14 (10) 1648-56. , XP000886521 abstract discussion --- -/--	16-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 May 2000	24/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Pilling, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	MURRY C E ET AL: "Macrophages express osteopontin during repair of myocardial necrosis." AMERICAN JOURNAL OF PATHOLOGY, (1994 DEC) 145 (6) 1450-62. , XP000886530 discussion abstract	16-20
Y	GIACHELLI, CECILIA M. ET AL: "Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo" AM. J. PATHOL. (1998), 152(2), 353-358 , XP000886540 discussion abstract	16-20
Y	DAOUD A S ET AL: "Sequential morphologic studies of regression of advanced atherosclerosis." ARCHIVES OF PATHOLOGY AND LABORATORY MEDICINE, (1981 MAY) 105 (5) 233-9. , XP000886554 abstract final paragraph of the comment on page 239	16-20
A	WO 92 22316 A (PHILADELPHIA CHILDREN HOSPITAL ;UNIV PENNSYLVANIA (US); UNIV CAMBR) 23 December 1992 (1992-12-23) page 9, line 21 -page 9, line 24 page 13, line 21 -page 13, line 23	1-32
A	US 5 695 761 A (HECK DIANE ELAINE ET AL) 9 December 1997 (1997-12-09) column 4, line 26 -column 4, line 27 column 4, line 43 -column 4, line 46	1-32
A	EP 0 705 842 A (HOECHST AG) 10 April 1996 (1996-04-10) abstract	1-32

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/29173

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU 2186392 A	12-01-1993
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EP 0705842 A	10-04-1996	AU 695117 B	06-08-1998
		AU 3308695 A	18-04-1996
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		JP 8191693 A	30-07-1996
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